Functional Dissection of Phagocytosis in Nervous System Development
and the Immune System of *Drosophila melanogaster.*

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Dipl. biol. Sofia Axelrod

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Jan-Hendrik Olbertz

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I
Prof. Dr. Andreas Herrmann

Gutachter/innen:
1. Prof. Dr. Harald Saumweber
   Humboldt University Berlin, Institute of Biology - Cytogenetics
2. Prof Dr. Ulrike Gaul
   Alexander von Humboldt-Professor
   Gene Center of the LMU, Department of Biochemistry
3. Prof. Dr. Salim Seyfried
   MDC

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Для моих любимых родителей

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Abbreviations

AF	Alexa Fluor
AMP	Antimicrobial Peptide
ANN	Annexin
C. elegans	*Caenorhabditis elegans*
CD	Cluster of Differentiation
cDNA	Complimentary Deoxyribonucleic Acid
CNS	Central Nervous System
CRC	Calreticulin
CRQ	Croquemort
D. melanogaster	*Drosophila melanogaster*
DNA	Deoxyribonucleic Acid
DRPR	Draper
dsRNA	Double Stranded Ribonucleic Acid
DSCAM	Down Syndrome Cell Adhesion Molecule
E. coli	*Escherischia coli*
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMI/ EMILIN	Elastin Microfibril Interface Located Protein
ER	Endoplasmatic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal Calf Serum
FDR	False Discovery Rate
FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein
IMD	Immune Deficiency
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
lof	Loss Of Function
LPC	Lysophosphatidyl Choline
LRP	Low Density Lipoprotein Related Protein
MBL	Mannan-Binding Lectin
MCR	Macroglobulin Complement- Related
MDR65	Multidrug Transporter 65
MMP	Matrix Metalloproteinase
NIM	Nimrod
OTE	Off- Target- Effects
ox	oxidized

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
p.	Page
PGRP	Peptidoglycan Recognition Protein
PGRP-LC	Peptidoglycan Recognition Protein Long Chain
PGRP-SA	Peptodiglycan Recognition Protein Short Chain
PI3K	Phosphatidylinositol-3-Kinase
PRR	Pattern Recognition Receptor
PS	Phosphatidylserine
qRT-PCR	Quantitative Real Time Polymrache Chain Reaction
RNAi	Ribonucleic Acid Interference
RT-PCR	Real Time Polymerase Chain Reaction
S. aureus	*Staphylococcus aureus*
SANTA MARIA	Scavenger receptor Acting in Neural Tissue And Majority of Rhodopsin Is Absent
SEM	Standard Error of the Mean
SHARK	SH2 Ankyrin Repeat Kinase
SIMU	Six Microns Under
siRNA	Short Interfering Ribonucleic Acid
Src	Rous Sarcoma oncogene Cellular homolog
SYB	Synaptobrevin
TEP	Thioester- Containing Protein
zCOP	Zeta Coat Protein

Abstract

Phagocytes remove apoptotic cells during development and eliminate pathogens in the immune system. The underlying molecular and cellular mechanisms, particularly the differences between macrophages and non-professional phagocytes like glia, are not well understood. We used novel cell-based assays to screen phagocytic function of candidate genes assembled from literature and our genome-wide transcription profiling of *Drosophila melanogaster* embryonic glia.

Gene function was knocked-down by RNAi and phagocytic efficiency assessed by flow cytometry; to explore functional specificity, we offered not only bacteria, but also apoptotic cells and beads as 'food'. To validate results *in vivo*, we analysed glial clearance of apoptotic neurons in embryonic development and immune clearance of bacteria in adult flies using both genetic mutants and transgenic RNAi.

Our screen provides a cross section of the different steps of phagocytosis from recognition to engulfment and phagosomal degradation. For the recognition of apoptotic cells, we confirm the involvement of known factors, such as the chaperone Calreticulin and phosphatidylserine-binding Annexin, and identify new players, such as NIMA for macrophage and SANTA MARIA for glial corpse clearance. We find components associated with vesicular trafficking including the v-SNARE Synaptobrevin and the cytochrome Cyp4g15 to be required for corpse clearance. Unexpectedly, receptors known for bacterial recognition, such as PGRP-LC and TEP2, are also strongly required for apoptotic clearance. Conversely, receptors previously implicated in apoptotic cell recognition are also required in bacterial clearance (SIMU, Draper), revealing cross-specificity of the system. Our work represents the first systematic and comparative assessment of the molecular repertoire of different types of phagocytosis, and, with the identification of many new players, lays the groundwork for a mechanistic dissection of bacterial and corpse clearance by glia and macrophages.

Zusammenfassung

Während der Entwicklung eines Organismus entfernen Phagozyten apoptotische Zellen, aber sie beseitigen auch Krankheitserreger im Immunsystem. Die zugrundeliegenden molekularen und zellulären Mechanismen, insbesondere die Unterschiede zwischen Makrophagen und nicht-professionellen Phagozyten wie Gliazellen, sind weitestgehend unklar. Wir haben neuartige Zellkultur-basierte Assays entwickelt, um die Phagozytosefunktion von 86 Kandidatengenen zu testen, die wir aus der Literatur sowie unserem Expressions-Profiling in embryonalen Gliazellen von *Drosophila melanogaster* zusammengestellt haben.

Die Genfunktion wurde durch RNAi herabgesenkt und die Phagozytoseeffizienz wurde mittels Durchflusszytometrie untersucht; um die funktionelle Spezifität der Gene zu erkunden, haben wir nicht nur apoptotische Zellen, sondern auch Bakterien und mikroskopische Kügelchen als „Essen“ angeboten. Mit Hilfe von Null-Mutanten und transgenem RNAi wurden die Ergebniss *in vivo* validiert. Um die Phagozytose apoptotischer Zellen testen, haben wir untersucht, wie Makrophagen und Gliazellen tote Zellen während der Embryonalentwicklung entfernen, während zur Untersuchung der bakteriellen Phagozytoze adulte Fliegen mit Bakterien infiziert wurden.

Unser Screen liefert einen Querschnitt durch die verschiedenen Schritte der Phagozytose von der Erkennung und Einverleibung bis zum phagosomalen Abbau. In Bezug auf die Erkennung von apoptotischen Zellen reproduzieren wir die Beteiligung von bekannten Faktoren, wie zum Beispiel des Chaperons Calreticulin und des Phosphatidylserin-bindenden Annexins, andererseits identifizieren wir neue Akteure wie NIMA für Makrophagen und SANTA MARIA für Gliazellen. Außerdem zeigen wir, dass Vesikeltransportkomponenten einschließlich der v-SNARE Synaptobrevin und des Cytochroms Cyp4g15 für das Fressen apoptotischer Zellen erforderlich sind. Überraschenderweise werden Rezeptoren wie PGRP-LC und TEP2, die für die Erkennung von Bakterien zustaendig sind, auch für die Phagozytose von apoptotischen Zellen benötigt. Umgekehrt sind Faktoren, die als Rezeptoren für apoptotische Zellen bekannt sind (SIMU, Draper), auch für die Phagozytose von Bakterien notwendig, wodurch eine grundlegende Kreuz-Spezifität des Systems zutage tritt. Unsere Arbeit liefert die erste systematische und vergleichende Analyse des molekularen Repertoirs der verschiedenen Phagozytosearten. Durch die Identifizierung vieler neuer Faktoren legt diese Arbeit

den Grundstein für ein mechanistisches Verständnis der Phagozytose von apoptotischen Zellen und Bakterien durch Makrophagen und Gliazellen.

1 Introduction

1.1 Evolution of phagocytosis, function in development and immunity

Phagocytosis is an evolutionarily ancient process used by unicellular organisms (protozoa) including amoebas and ciliates for nutrient uptake (Dzik 2010). In multicellular animals, single cells proliferate and differentiate into organisms, consisting of specialized cell types carrying out different functions like digestion and reproduction. However, during these processes of cell division and proliferation mistakes occur and unwanted cells are created. These superfluous cells kill themselves through programmed cell death/ apoptosis for the greater good of the organism (Koonin & Aravind 2002). Multicellular organisms are also exposed to the constant threat of exploitation by parasitic microorganisms that invade the animal. What happens to the dying cells and the invading pathogens? Metazoans need to remove extra cells formed during development and differentiation, as well as senescent cells and cells damaged or altered by pathological processes such as trauma, cancer or infection. As organisms became multicellular, it was therefore necessary to have a system of self-surveillance in place to ensure proper development and homeostasis. This task of removing dangerous self and non-self from the organism is carried out by eating cells called phagocytes, and freely moving cells with phagocytic features are present in all multicellular organisms (Danilova 2006; Dzik 2010; Hartenstein 2006). In the simplest metazoans, sponges and cnidaria, amoebocytes and interstitial cells, respectively, eat apoptotic cells and pathogens – but these cells also perform other functions including digestion and gametogenesis (U. Technau et al. 2003; David et al. 2005; Agnello & Roccheri 2010; Custodio et al. 1998). With the evolution of higher body plans and the complex developmental generation of different cell types, the demand for apoptotic removal and tissue homeostasis increased manifold. Microorganisms that specifically infect and damage host animals also coevolved simultaneously. This arms race between hosts and microorganisms, as well as the increasing developmental and homeostatic load of apoptotic/ damaged/ altered cells, necessitated an increasingly sophisticated immune system with more specialized and efficient phagocytes that could sense, find and engulf a target, and therefore distinguish between healthy self, dangerous self and non-self. While these phagocytes are called professional, many other cell

types retained or secondarily regained the ability to phagocytose; these cells are the so called non-professional phagocytes, which do not actively search for a target, but, if in the vicinity of a dying cell or a pathogen, can perform the task of eating (Williams-Herman & Werb 1999; Rabinovitch 1995; Gregory & Pound 2011). Research in two of the major genetic animal models, the nematode worm *C. elegans* and the fruit fly *Drosophila melanogaster* provided important insight into the mechanisms and evolution of phagocytosis. In *C. elegans*, development is highly deterministic and the number of cells that undergo apoptosis is exactly predetermined: 131 cells die in hermaphrodites and 147 in male animals. Since *C. elegans* does not possess professional phagocytes, it is the neighbors of the dying cell that engulf the corpse (Gumienny & Hengartner 2001). In contrast, *Drosophila* does possess professional phagocytes, which are called haemocytes and are very similar to vertebrate macrophages (Lemaitre & J. A. Hoffmann 2007). What do these phagocytes eat? Nervous system development is a process with particularly high apoptosis rates, and the proportion of dying cells increases continuously throughout evolution: while almost no cells die during nematode development, around 50% of neurons die in the *Drosophila* central nervous system (CNS), and up to 80% of neurons kill themselves in vertebrates, because they have not received the right survival signals, not migrated to the right place or not innervated the right tissue (Buss et al. 2006; Kuan et al. 2000; Rogulja-Ortmann et al. 2007). To create the functional complexity of a healthy brain it is particularly important to efficiently remove unwanted cells, and the *Drosophila* embryo has proven as an excellent model to study these processes (Kinchen 2010). At early stages of embryonic development it is macrophages that cruise the embryo end efficiently remove corpses, however the CNS becomes ensheathed by the blood-brain-barrier at embryonic stage 16, after which macrophages can no longer enter the CNS (Schwabe et al. 2005). However, cell death in the CNS peaks at these late stages of embryonic development – many neurons die and have to be efficiently removed in order not to impede proper development. It is a CNS-resident cell type, the cell body or cortex glia (hereinafter ‘glia’) that performs this phagocytosis task and is solely responsible for clearing dying neurons in the CNS. These non-professional phagocytes are, in contrast to macrophages, not motile but remain stationary at their CNS positions, probing their surroundings for apoptotic cells with the help of protrusions like filo- and pseudopodia (Kurant et al. 2008).

1.2 Phagocytosis is a three step process

Regardless of whether apoptotic cells or pathogens are being cleared, and whether macrophages, glia or other cell types perform the task, phagocytosis has some uniform steps and features present in all its instances (Stuart & Ezekowitz 2005). Phagocytosis occurs in three major steps: recognition, engulfment and degradation (**figure 1**).

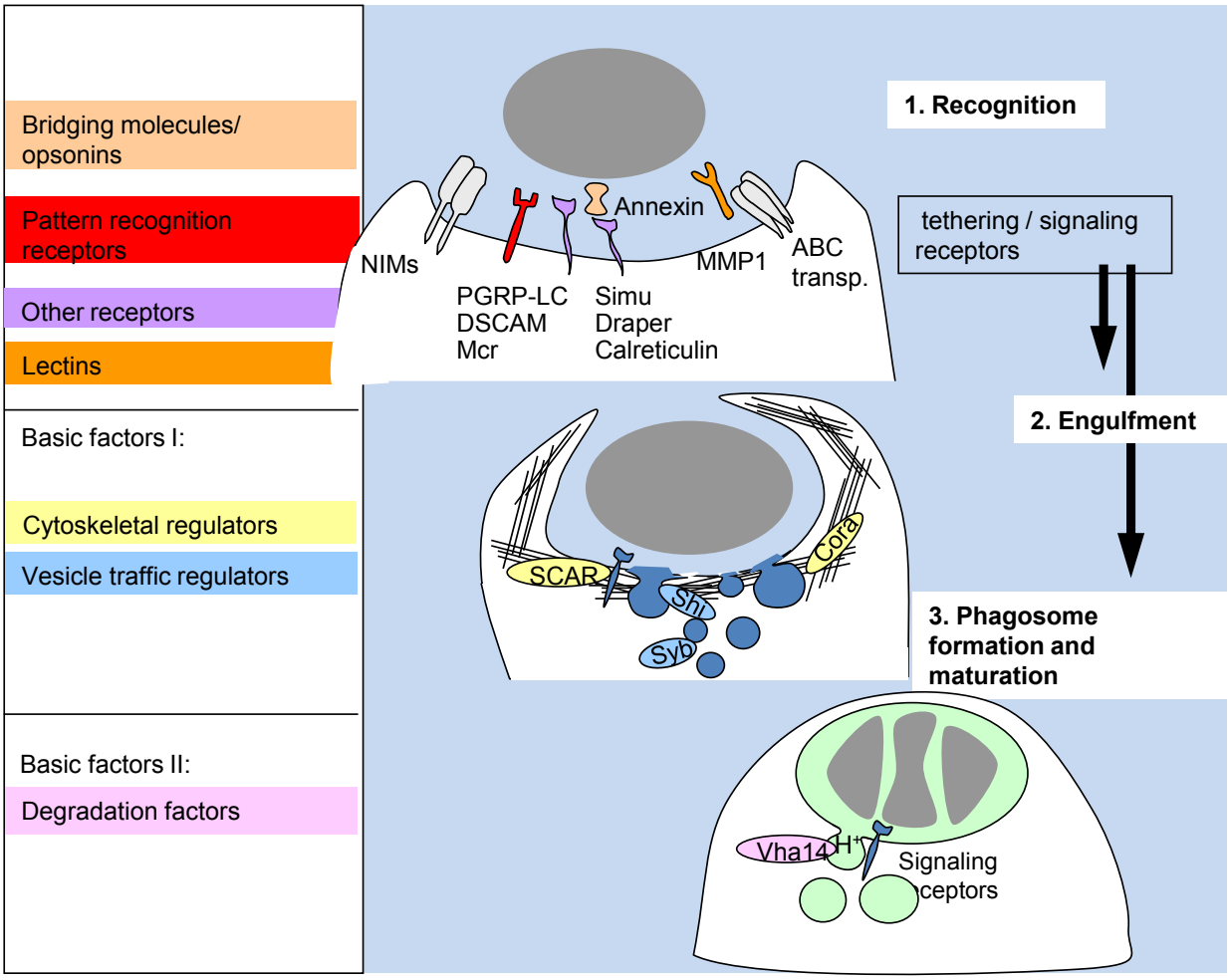


Figure 1: Phagocytosis of any particle occurs in three steps.

Depicted are the three steps of phagocytosis - recognition, engulfment and phagosome maturation - as well as some of the corresponding factors that have been investigated in the present work.

The recognition step requires the phagocyte to sense its target, which can occur via plasma membrane receptors binding to ligands on the target particle, or secreted bridging molecules/ opsonins labeling target particles for phagocyte recognition. A third mechanism completely independent of specific receptor-ligand interactions is macropinocytosis, or cell drinking. Once a target particle is recognized,

transmembrane signaling activates cytoskeletal components to form protrusions that enwrap the target in a phagocytic cup. Simultaneously, vesicular trafficking components deliver vesicles to the phagocytic cup to extend its surface. Eventually, the protrusions fuse and a phagosome is formed. Interactions with the endocytic and lysosomal pathways cause hydrolases and proton pumps to be delivered to the phagosome, which acidifies and acquires degradative characteristics. Finally, the phagosomal content is completely dissolved and absorbed by the eating cell.

1.3 Phagocytic target recognition

1.3.1 Pathogen receptors

Even amoeba express phagocytic receptors, lectins, to sense specific sugar residues on the surface of bacteria, and most phagocytic receptor subtypes are present in all metazoans (Danilova 2006). In multicellular animals, correct recognition of a phagocytic target is crucial for development and maintenance of a healthy organism, and a phagocyte therefore needs to identify phagocytic targets amongst healthy cells that ought not to be touched. It then needs to distinguish between the types of targets it encounters. If the target is a pathogen or otherwise dangerous, e.g. a cancerous cell, engulfment needs to be followed by the initiation of an immune response in order to alarm the organism about the dangerous objects. However, if the target is an apoptotic or damaged cell, phagocytosis has to remain ‘silent’ without evoking any proinflammatory signaling. Inflammation in response to apoptosis is highly undesirable, since it leads to autoimmune reactions and diseases (Krysko et al. 2006). The surface features on the target particles are recognized by receptors on the phagocytes, enabling them to distinguish between the various target particles. The receptors are called pattern recognition receptors (PRRs) and identify repetitive molecular patterns on the target surface (Janeway & Medzhitov 2002). These receptors can be either membrane-bound or secreted, in which case they are called opsonins. Certain PRRs do not (exclusively) promote phagocytosis, but instead function as signaling receptors to transduce information about the recognized particle. What are the molecular patterns recognized by PRRs? A lot of the research investigating the mechanics of this process

has been modeled on vertebrate cultured cells, and many factors have been implicated in each of the three steps of phagocytosis (Hamon et al. 2006; Gardai et al. 2005; Hamon et al. 2006; Oka et al. 1998). Early cell culture studies provided first

insight into the basic principles underlying pathogen phagocytosis through two paradigmal phagocytosis types: phagocytosis through the Fc-receptor for antibody-opsonized and the integrin/ complement receptor for complement-opsonized pathogens. Upon ligation, both receptor types initiate a signaling cascade leading to engulfment and target degradation. These cell culture studies for the first time investigated the process of phagocytosis and revealed the molecular players involved in the different steps of the process, and in particular shed light on the interaction between phagocyte and (bacterial) target. Bacterial cell walls contain highly ordered sugar structures like mannan, lipopolisaccharides or proteoglycans that distinguish them from eukaryotic cells. These structures are recognized by different secreted and membrane-bound molecules including collectins, complement, peptidoglycan-recognition proteins (PGRPs), CD14 and mannan-binding lectin (MBL). Opsonins like MBL and complement label particles for engulfment and are recognized by integrin-based complement receptors on phagocytes. Yet other receptors like scavenger or toll-like receptors directly interact with the particle surface and trigger engulfment. Knockout mice for most of the discovered phagocytosis receptors confirmed the crucial function of each individual opsonin and receptor: mice deficient in the Fc receptor, CD14, complement receptor, MBL, complement components, surfactant proteins and the macrophage mannose receptor are more susceptible to different infections due to their impaired innate immunity and their defunct pathogen phagocytosis (Haziot et al. 1998; Ip et al. 2008; Ravetch & Clynes 1998; P. R. Taylor et al. 2007; Devitt et al. 2004).

Despite the long coevolution of microorganisms, recognition of these molecular patterns on microbes has remained a major mechanism of innate immunity. This is probably due to the fact that these receptors have multiple ligand-binding domains, which individually bind their ligands only with low affinity. Only recognition and binding of repetitive molecular structures, as it is the case on the highly ordered bacterial cell walls, promotes engulfment. This synergistic effect enables recognition and engulfment even when the microorganism modifies its surface structure to evade immune recognition. Also, because multiple receptors need to be engaged by a polymer structure, autoimmune phagocytosis of healthy cells is minimized (Janeway et al. 1996).

1.3.2 Apoptotic cell receptors

Similar to microorganisms, apoptotic cells display surface patterns including proteins, lipids and sugar residues that are not present on normal cells. Research on

phagocytosis of apoptotic cells is a more recent development, but many factors have already been found to play a role in the process. Mammalian studies investigating individual genes required for phagocytosis of apoptotic cells have been studied in pure cell culture studies as well as *in vivo*. Interestingly it became clear early on that a major factor in the process of apoptotic cell recognition is not a protein, but a lipid. By inhibiting the interaction between phagocyte and apoptotic cell through phosphatidylserine (PS)-containing liposomes it was demonstrated that PS, a negatively charged phospholipid, is involved in apoptotic cell phagocytosis (Fadok et al. 1992). PS is restricted to the inner leaflet of the plasma membrane in healthy cells, but becomes rapidly exposed to the outside of the cell during apoptosis, thereby promoting engulfment. PS is the most prominent feature and marker of apoptotic cells and has been shown to be recognized by different opsonins including ANNEXIN, GAS6 and MFGE8 as well as the phagocyte receptors MER, complement receptor ($\alpha_v\beta_3$-integrin) and BAI1 both *in vitro* and *in vivo* (Ravichandran & Lorenz 2007; Y. Wu et al. 2006; Fadeel 2004). Phospholipids not only get redistributed in apoptotic cells, but also oxidized by reactive oxygen species, which are increasingly produced in the dying cell (Tyurina et al. 2000; Leitinger 2003). These oxidized lipid species need to be recognized by phagocytes, and the scavenger receptor CD36 has been shown to bind different oxidized phospholipids on apoptotic cells and promote phagocytosis by macrophages, dendritic cells and neutrophils (Albert et al. 1998; R. L. Silverstein & Febbraio 2009). Furthermore, *cd36* knockout mice have been shown to contain lingering apoptotic cells due to impaired phagocytic removal (Greenberg et al. 2006). Lipids play an important role in the phagocytosis of apoptotic cells not only as “eat-me”, but also as “find-me” signals: the phospholipid lysophosphatidylcholine (LPC) had been implicated in phagocyte attraction to the apoptotic cell through inhibition of LPC-generating phospholipase A in apoptotic cells and transmigration assays using synthetic LPC (Mueller et al. 2007). The lipids PS and LPC are some of the earliest markers of apoptotic cells and should lead to rapid identification and engulfment by phagocytes. If, however, elimination of early apoptotic cells fails, the apoptotic cell shrinks and exposes normally endoplasmaic reticulum (ER)-resident molecules including the chaperones Calreticulin (CRC) and Calnexin as well as immature glycoproteins and glycolipids, which have been shown to be required for phagocytic recognition in cell-culture as well as genetic knockout studies in mice (Gardai et al. 2005; Franz et al. 2006). Lectins on phagocytes including C1q, MBL and surfactant proteins A and D have been initially implicated in bacterial clearance, but they also bind newly exposed glycosyl residues on apoptotic

cells and contribute to apoptotic clearance as well (Ogden & Elkon 2006; Ogden et al. 2001; Stuart et al. 2005). Apoptotic cells that have not been engulfed at this point undergo secondary necrosis, which can lead to the cell's contents spilling into the extracellular space. In necrotic death, the orderly process of corpse elimination is abandoned. Intracellular epitopes become exposed, bind to immune receptors and thereby turn into autoantigens, which can lead to autoimmune reactions (Krysko et al. 2006; Y. Wu et al. 2006). In contrast, apoptotic cells are engulfed without initiating an immune response, but rather lead to immunosuppressive signaling and release of anti-inflammatory cytokines (Fadok et al. 1998; Voll et al. 1997; Henson 2005).

Many receptors contribute to phagocytosis of pathogens and apoptotic cells, and some are even shared amongst each other, for example *cd14*, *cd36* as well as complement receptors (Siamon 2002). Phagocytosis of pathogens and necrotic cells leads to expression of immune genes like antimicrobial peptides (AMPs) or cytokines, while corpse engulfment remains an immunologically silent event. How does a cell, if it employs the same receptors for both types of phagocytosis, know when to engage an immune response? One way that has evolved in vertebrates is separating the detection of pathogen associated molecular patterns and phagocytosis. Vertebrate Toll-like receptors do not promote engulfment, but specifically recognize foreign lipopolysaccharide, DNA, RNA and other molecular patterns of dangerous invaders and initiate immune signaling. On the other hand, there is some overlap between recognition and signaling, as at least CD14 and MBL serve as both phagocytosis as well as signaling receptors. It has been shown that both receptors promote pro-inflammatory signaling when binding pathogens, but not apoptotic cells (Jiang et al. 2005; Ip et al. 2008). How the same phagocytic receptors can do both, elicit immune responses when binding to a pathogen and remain silent when binding to an apoptotic cells, remains to be elucidated, but is thought to be a result of combinatorial input of different receptor-target interactions.

1.4 Studying phagocytosis in genetic model organisms

In vivo mouse studies have provided insight into the process of corpse clearance, and while some of these studies observed lingering apoptotic cells in the knockout mice, other studies were hampered by severe phenotypes like developmental defects and autoimmune diseases (Lu & Lemke 2001; Kunisaki et al. 2004; Bader et al. 1998; Park et al. 2004; Kunisaki et al. 2004; Roszer et al. 2011; Dahl et al. 2003; Koyasu 2003). Although these phenotypes are believed to be consequences of failed

corpse removal, in many instances a direct proof is missing. Another difficulty in genetic knockout studies is genetic redundancy and pleiotropy, as can be seen in the instance of integrins. Integrin involvement in phagocytosis of apoptotic cells has been shown in cell culture already in 1990 (Savill et al. 1990). Later it has been shown that genetic knockout of the alpha 5 subunit is perinatally lethal, because the gene is involved in early processes of morphogenesis (Bader et al. 1998). On the other hand, knockout of the beta 5 subunit does not show any developmental defects, and only old mice develop age-related blindness due to defective phagocytosis of apoptotic retinal cells (Nandrot et al. 2004).

Due to this complexity of the organism and high redundancy of factors in vertebrates, genetic invertebrate models including *C. elegans* and *Drosophila* have proven particularly useful in isolating individual factors. Redundancy is most likely attributable to two rounds of genome duplications at the base of the vertebrate lineage (Kasahara 2007). The resulting tetraploidization allowed functional diversification and redundancy of genes, which facilitate complex body plans, but hamper studies trying to understand the function of a specific gene (Makałowski 2001). However, most gene families present in vertebrates already exist in invertebrates, but diversification and gene duplication have not occurred yet, so that these gene families in invertebrates consist only of few or even just one member carrying out a specific function. For example, the serine protease pathway triggering apoptosis/ programmed cell death has sufficient redundancy in vertebrates such so that knockout of individual constituents of the pathway, the caspases, does not lead to any phenotypes in mice (Lockshin & Zakeri 2004; Lakhani et al. 2006). In *C. elegans*, however, where the genes governing programmed cell death were initially discovered, there are only single genes for each functional factor in the genome, and knocking out each of these genes leads to the generation of easily detectable additional cells that are normally eliminated by apoptosis (Ellis & Horvitz 1986; Lettre & Hengartner 2006). It was also in *C. elegans* that a genetic screen for genes affecting removal of apoptotic cells uncovered two partially redundant signaling cassettes (Zhou et al. 2001; Gumienny & Hengartner 2001; Gumienny et al. 2001; Reddien & Horvitz 2004). Knockout of individual factors from both pathways increased the number of unengulfed corpses, and removal of factors from either pathway produced an even stronger effect. However, residual phagocytosis was still observed in these double knockout mutants, pointing at additional mechanisms facilitating phagocytosis (Kinchen & Hengartner 2005).

Table 1: Comparison of methods of RNAi phagocytosis screens in S2 cells.

	Ramet 2002	Philips 2005	Kocks 2005	Strosch.-Steve. 2006	Axelrod 2011
Plates [wells]	48	384	48	96	96
dsRNA/ cell [µg]	8	1.5	2	2	0.7
t RNAi [days]	2.5	3	3	4	3
Bacteria	heat-killed FITC-*E. coli*	heat-killed FITC-*E. coli*	heat-killed FITC-*E. coli*	live GFP-expressing *E. coli*	heat-killed AF488-*E. coli*
Eating Synchronization	30 min ice	spin	30 min ice	no	spin
E. coli eating time	15 min	45 min	15 min	2 h	2.5 h
S. aureus eating time	20 min	-	20 min	2 h	3 h
Beads eating time	-	-	-	20 h	2 h
TrypanBlue	yes, pH 5.5	yes, pH 4.4	yes, pH 5.5	no	yes, pH 5.5
Fixation	no	no	no	yes	no
Stainings	-	Hoechst	-	Hoechst, α-*E. coli*	-
Read-out	FACS	Fluorescence reader	FACS	Microscopy	FACS
# of genes tested	1000	12000	45	7000	86
Secondary screening	no	no	no	resynthesis of probes for 184 hits	secondary RNA for 19 genes
# of experiments (n)	2-5	3	3	1	3-7
SEM *E. coli*	14.13	11.09	8.36	-	4.97
SEM *S. aureus*	12.67	-	4.66	-	3.96
SEM beads	-	-	-	-	3.12

Shown are different parameters of the protocols for RNAi treatment and phagocytosis assays.

1.5 Phagocytosis in *Drosophila*

1.5.1 Phagocytosis screens

In *Drosophila* the advent of RNAi and the simple bathing transfection method precipitated a number of screens for phagocytic function, which were carried out in S2 cells, an embryonic macrophage cell line (Stroschein-Stevenson et al. 2009; Rämet et al. 2002; Philips et al. 2005; Agaisse et al. 2005; Ulvila et al. 2011). All of these screens were aiming at the discovery of novel factors in phagocytosis of pathogens including *S. aureus*, *E. coli*, mycobacteria, listeria and yeast. Multiple screens used *E. coli* and *S. aureus*, therefore allowing for a comparison of the screen results. The overlap between the screens is low (**figure 2, p. 20**). When looking at the few genes that were found in more than one screen, for 76% of the hits the actual effect strengths are significantly different from each other. These discrepancies are likely due to differences in protocols. Between all studies all of the relevant parameters differed: dsRNA concentration, duration of RNAi treatment, type of phagocytic targets and detection method (**table 1, p. 18**). Still, these studies found a number of genes involved in different types of phagocytosis, such as *eater* and *pgrp-lc* for *E. coli*, *mcr* for yeast and *peste* for *Mycobacterium marinum*.

1.5.2 *in vivo* phagocytosis studies in *Drosophila*

Even though these cell-based screens identified a number of novel factors specifically required for phagocytosis of each pathogen, *in vivo* validation has been shown only for a small subset of the genes found (Rämet et al. 2002; Bou Aoun et al. 2011; Kocks et al. 2005). In terms of phagocytosis of apoptotic cells, no screens have been published so far, but a small number of in-depth studies elucidated the function of individual molecules in the *in vivo* process. The first *Drosophila* receptor for apoptotic cells discovered is a homolog of the vertebrate CD36 scavenger receptor, which had previously been implicated in the recognition and phagocytosis of both bacteria and apoptotic cells in vertebrates (Greenberg et al. 2006). Its ortholog in *Drosophila*, *croquemort* (*crq*), is expressed in embryonic macrophages and required for phagocytosis of apoptotic cells in the developing embryo, illustrating the conservation of phagocytic factors throughout evolution (Franc et al. 1996; Franc, Heitzler, et al. 1999). Recently a novel class of 13 mostly clustered secreted and transmembrane receptors was identified in the *Drosophila* genome, the NIM gene family (Kurucz et al. 2007; Somogyi et al. 2008). Members of the NIM family are characterized by a N-terminal cysteine-rich EMI or EMI-like domain, which was first characterized in the EMILIN family of glycoproteins of the extracellular matrix (ECM) (Doliana et al. 2000).

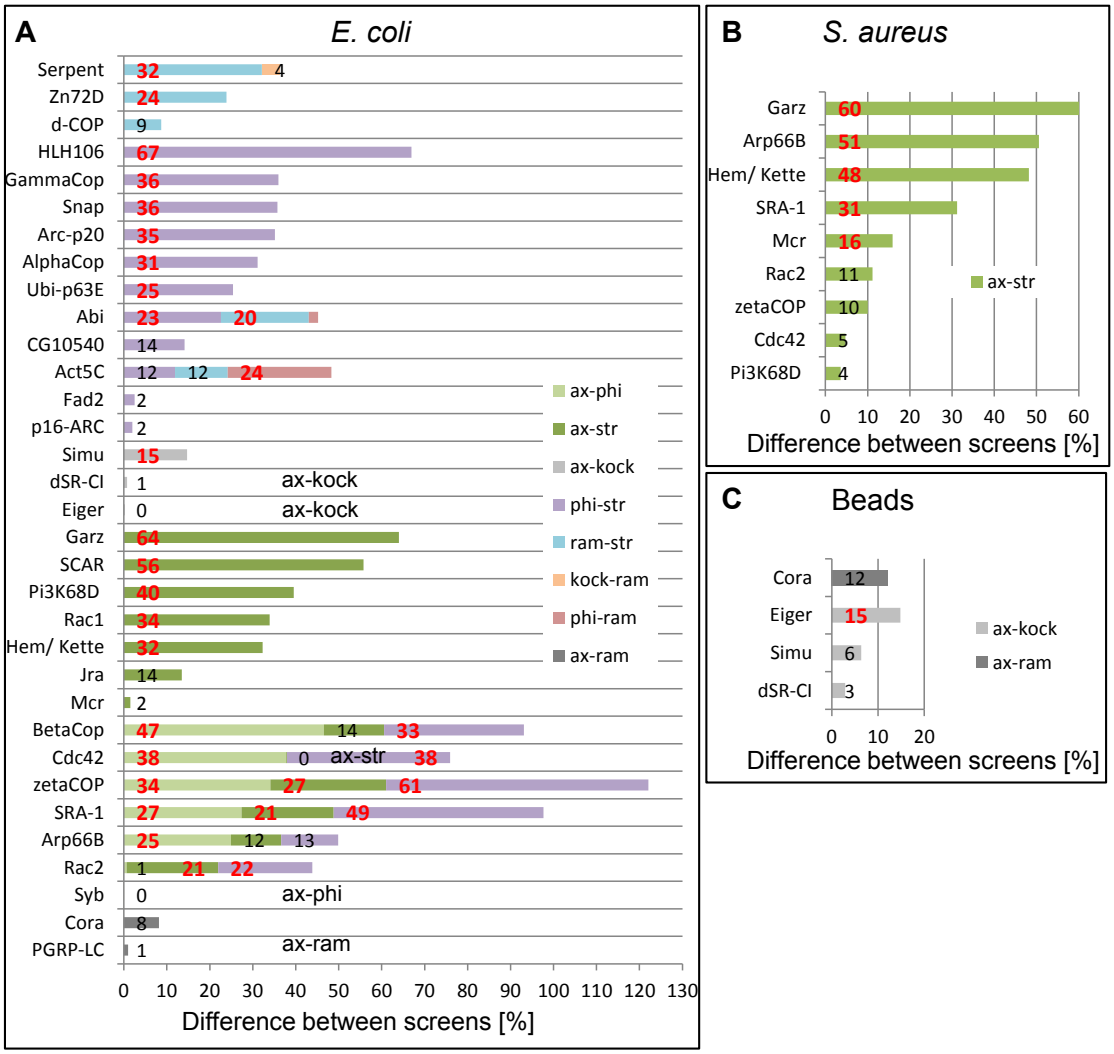


Figure 2: Overlap between S2 cell phagocytosis screens.

34 genes have been shown to have an effect on a particular type of phagocytosis in at least two different phagocytosis screens. Shown are the differences between the results of different phagocytosis screens for A E. coli, B S. aureus and C beads for RNAi knock downs of different genes. The colored bars represent the difference between results of two screens for a specific gene, the bigger the bar, the higher the discrepancy. Bar values are shown. Significant differences (>15%) are shown in red. 100% difference would mean that RNAi of a given gene compared to the control leads to no reduction in phagocytosis in one screen, and 100% reduction of phagocytosis in another. E. coli phagocytosis has been most extensively studied, and some genes have been tested in three screens, in such instances the differently colored bars are stacked above each other and are represented by one bar. The compared screens are Ramet et al. 2002 (ram), Philips et al. 2005 (phi), Kocks and al. 2005 (kock), Stroschein-Stevenson et al. 2006 (str) and the work presented here (ax).

The EMI domain contains a highly conserved CCxGY motif at its C-terminal end. In members of the NIM family, the EMI domain is followed by one or multiple NIM-type

EGF domains. These two features constitute the core present in all NIM family members, and is followed by different types and repetitions of EGF domains (**figure 3, p. 22,** Kurucz et al. 2007). Members of the NIM superfamily can be found in many organisms throughout evolution in invertebrates and vertebrates, and while some of them have been implicated in phagocytosis, the function of most family members is unknown. The first member of this family to be described in *Drosophila* was Draper (DRPR), a transmembrane receptor containing 15 extracellular EGF-like repeats and an N-terminal EMI domain (M. R. Freeman et al. 2003). *drpr* is the fly ortholog of the known *C. elegans* phagocytosis receptor *ced-1* and shares sequence similarity with the mammalian phagocytosis receptor *mfg-e8*, both of which have been shown to play a role in apoptotic clearance (Naka et al. 2009; Zhou et al. 2001). DRPR turned out to be the first receptor identified on *Drosophila* glia and was shown to be required for phagocytosis of apoptotic cells in the developing embryo and larva, as well as in the adult brain for engulfment of dying neurons, axonal debris and degenerating axons after traumatic injury (MacDonald et al. 2006). *drpr* has an intracellular Src phosphorylation motif and interacts with the non-receptor tyrosine kinase SHARK for phagocytosis signaling (Ziegenfuss et al. 2008). Interestingly, as our lab showed recently, in *drpr* null mutants apoptotic material accumulates inside phagocytes in the embryo revealing that *drpr* in fact is not required for the recognition step of phagocytosis, but rather for phagosome maturation (Kurant et al. 2008). Finding *drpr* and identifying its function, for the first time put glia on the map as a phagocytosing cell type in *Drosophila*, and our lab's interest in glia prompted us to intersect our research on glia with questions about phagocytosis. To further elucidate the role of glia in embryonic development, our lab had performed an expression-based screen for genes differentially upregulated in embryonic glia (H. Courvoisier, D. L. J. Fak, N. Rajewsky, and U. Gaul, unpublished data). Different phagocytosis genes known from other model organisms were found, including members of both *C. elegans* signaling cassettes together with orthologs of factors from vertebrates, pointing to the important role glia play in CNS phagocytosis (**figure 4, p. 23**).

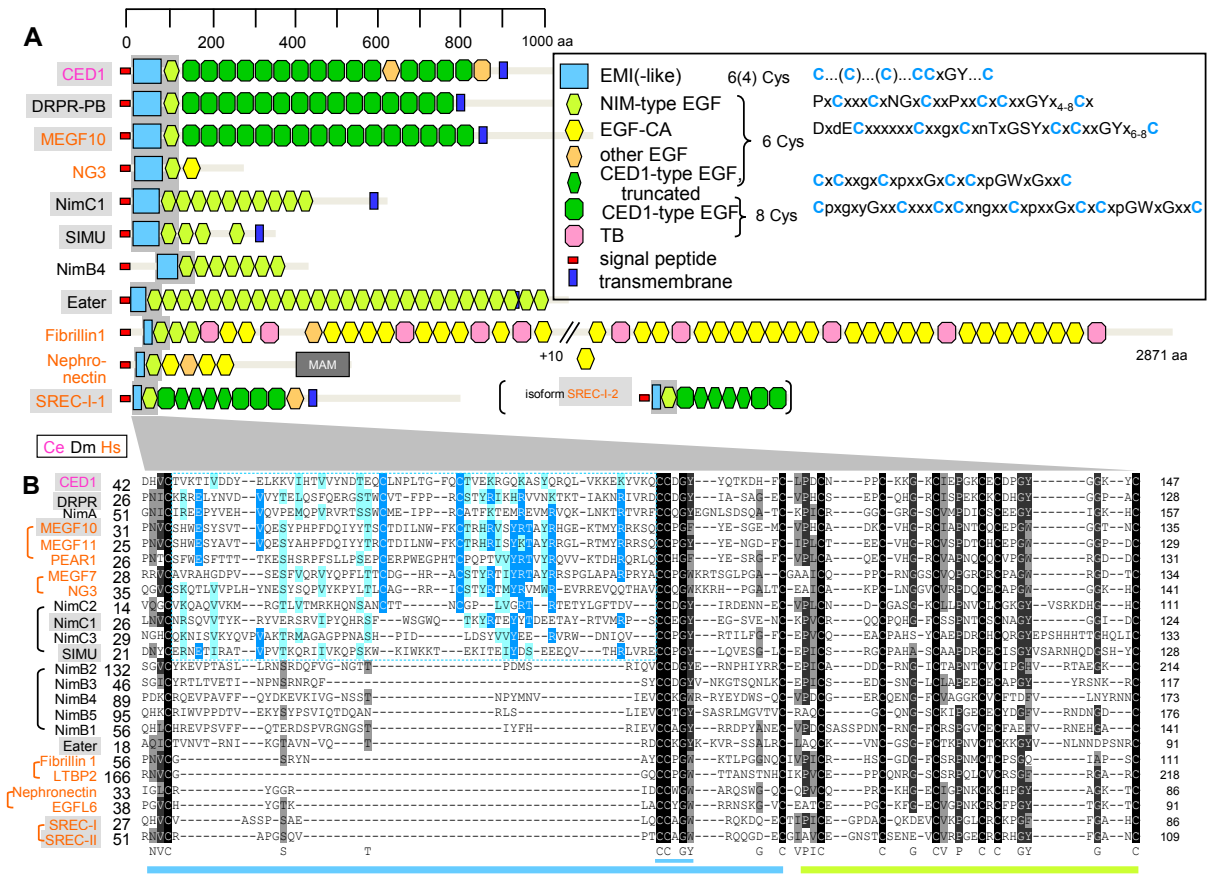


Figure 3: Comparison of EMI (-like) and NIM domain proteins

(from Kurant et al., Cell, 2008). Typical domain organizations of CED-1, its homologs, and other proteins containing an N-terminal EMI(-like) +NIM domain from worm, fly, and human; names of proteins with a demonstrated role in phagocytosis are highlighted in grey. Note that in most insect proteins the common EMI (-like)+NIM core is followed by additional NIM domains, while the vertebrate proteins contain tandem arrays of EGF-like repeats similar to those found in CED1 or other EGF-type domains. ***B*** *Sequence alignment of the EMI (-like) +NIM core for all known proteins containing this signature from worm, fly, and human. Proteins with similar domain organization as represented in* ***A*** *are grouped and bracketed; identical residues are boxed in black, similar residues in grey. Note that the EMI(-like) domains all share a highly conserved CCxGY motif at the C-terminal end of the domain, with invertebrate and vertebrate proteins showing different forms of internal truncation relative to the canonical EMI domain as represented by CED-1. A separate superimposed alignment (identical residues boxed in dark blue, similar residues in light blue) reveals the stronger similarity of the* Drosophila *NimC proteins, including SIMU, with the canonical EMI domain; although the two internal Cysteines are missing in the NimC proteins, other motifs are preserved.*

This profiling also recovered many novel genes. In particular another member of the NIM family, *simu*, was found to be highly expressed in glia, suggesting a similar role to *drpr*. *simu's* extracellular portion is shorter than *drpr's*, and in contrast to *drpr* it does not have an intracellular signaling domain. *simu*, like *drpr*, carries the canonical

N-terminal EMI domain, which is followed by three cysteine-rich NIM repeats. We found *simu* to be expressed in embryonic glia and discovered that it is required for uptake of apoptotic cells, which was dependent on the presence of its EMI domain, but not its transmembrane domain, suggesting that SIMU can function as an opsonin.

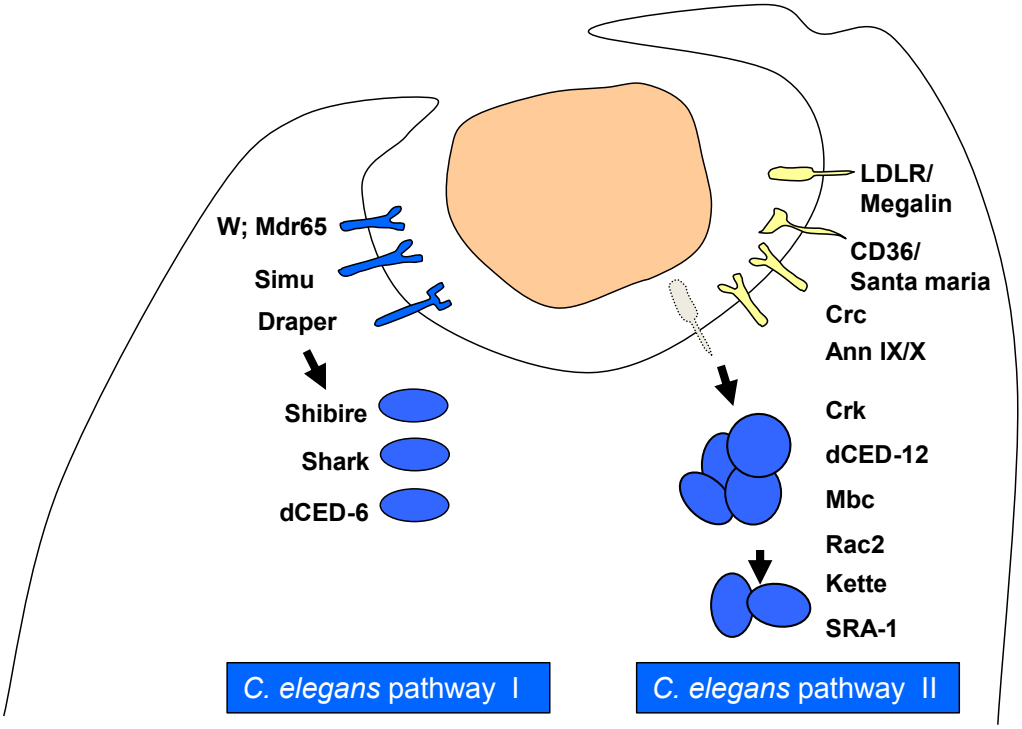


Figure 4: Glia possess the molecular repertoire for corpse engulfment.

Shown are homologs of known phagocytosis genes that are expressed or upregulated in Drosophila *embryonic glia, as revealed by affimetrix microarray analysis. Constituents of both* C. elegans *pathways have been recovered in glia (blue), as well as homologs to known vertebrate factors (yellow).*

Moreover, we found *simu* to function in the same pathway as *drpr* in corpse engulfment, with SIMU apparently being required for the recognition and DRPR for phagosome maturation signaling. Interestingly, both proteins are also expressed on macrophages and have similar functions there, underlining the molecular and functional similarities between both cell types (Kurant et al. 2008). However, *nim* genes are not only involved in apoptotic cell clearance; members of this family also play important roles in immunity. The macrophage receptor *eater* carries as many as 28 extracellular NIM repeats and does not have an intracellular signaling domain. Larval hemocytes from *eater* flies show less eating of *E. coli* and *S. aureus ex vivo*,

and adult flies are more susceptible to infection with the Gram-negative pathogen *Serratia marcescens*, pointing at *eater*'s role in bacterial phagocytosis (Kocks et al. 2005). Another member of the NIM family was recently identified and implicated in bacterial clearance: *nimC1*, which is similar to *simu* but contains more extracellular NIM repeats, and is required for phagocytosis of *S. aureus* by larval hemocytes (Kurucz et al. 2007).

These discoveries show that *Drosophila* is a suitable model to study the specific functions of phagocytes and their genes that are already highly specialized here, but not yet as redundant as in vertebrates. While the phenomenon of non-professional phagocytes including glia has been described in vertebrates, the cellular and molecular mechanisms of their apoptotic clearance remain unknown (Parnaik et al. 2000; Henson & Hume 2006; Hanayama & Nagata 2005). In *Drosophila*, glial function has been investigated in more depth, and it has become clear that glia are highly capable of phagocytosis and are equipped similarly to macrophages in order to perform the task, and current research tries elucidate the underlying molecular and cellular mechanisms of glial phagocytosis. Three paradigms have been used in the field: first, clearance of apoptotic cells in the embryonic nervous system, which yielded *simu* and *drpr*. Another paradigm is the pruning of degenerating axons in the third instar larval mushroom body during metamorphosis, which, apart from the involvement of *drpr* and the cytoskeletal adaptor protein *d-ced6*, revealed that the Ecdysone receptor is required for both apoptosis in the degenerating neurons as well as phagocytosis by the enwrapping glia (Awasaki et al. 2006; Awasaki & K. Ito 2004). Finally, engulfment of severed and degenerating axons after injury, called Wallerian degeneration, in the adult brain has been used as a model for glial phagocytosis, and *drpr* as well as its effector *shark* have been found to play a role here (MacDonald et al. 2006; Ziegenfuss et al. 2008). These studies increased our understanding of glia as phagocytes, but a lot remains to be learned about the mechanics of the process.

Drosophila macrophages have been predominantly studied in the context of immunological questions in cell culture or the adult animal (Brennan & Anderson 2004; Williams 2007) and are also poorly understood with regard to their function as corpse removers. Research in that area focused on embryonic clearance of corpses and has identified only few genes so far: in addition to *simu* and *drpr*, which are also present in macrophages, and the receptor *crq*, constituents of the calcium signaling cascade and proteasomal degradation pathway have been implicated in the process

(Cuttell et al. 2008; Silva et al. 2007). Taken together, our understanding of phagocytosis of both apoptotic cells and pathogens by glia and macrophages is limited, prompting us the conduct the screen presented here.

1.6 A novel phagocytosis screen

Our screen was motivated by the discovery that several known and many putative phagocytosis genes from *C. elegans* and vertebrate models were expressed or even upregulated in *Drosophila* embryonic glia, and the implication of the NIM family member *simu* in glial phagocytosis of apoptotic cells (**figure 4, p. 23**). *simu* was highly expressed in glia and turned out to be a novel receptor for apoptotic cells, solidifying the important role glia play in embryonic corpse clearance and prompting us to test other genes differentially expressed in glia for their putative involvement in the process.

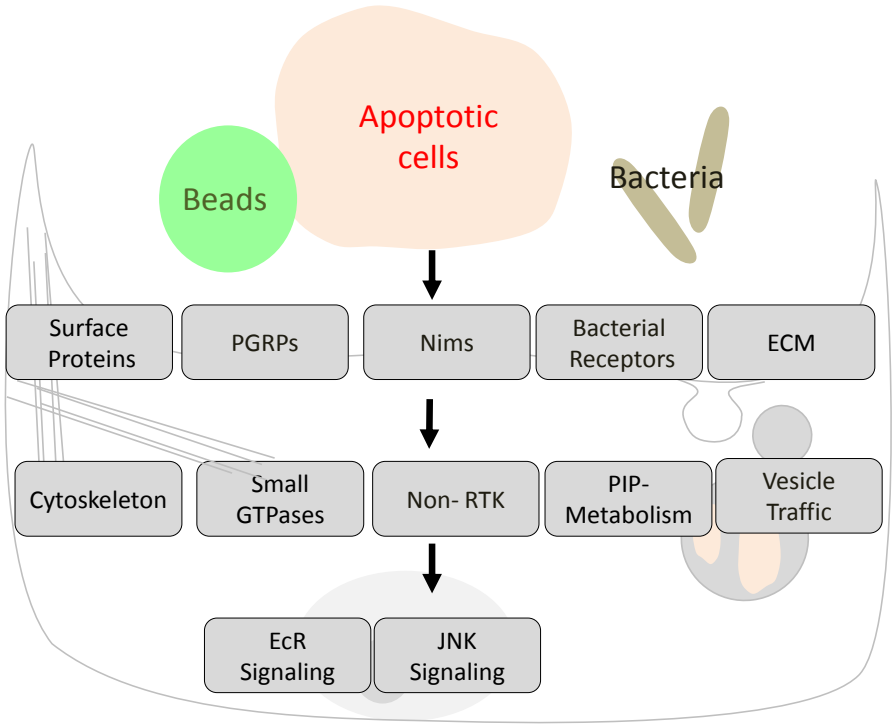


Figure 5: Phagocytosis screen - candidate categories and food types.

Our screen comprises candidates from different cellular compartments along a longitudinal cut through the cell. Tested food types include charged uncoated beads, apoptotic cells, S. aureus *and* E. coli *bacteria.*

As only *simu* and *drpr* had been implicated in apoptotic clearance in the *Drosophila* embryo so far, we wanted to identify additional players in the process to shed light on the function of glia as phagocytes. Our expression analysis combined with previous knowledge about these genes provided a useful pool of candidates potentially involved in glial phagocytosis.

A second motivation for our screen came from the fact that phagocytosis is involved in two different processes: clearance of endogenous dead material as well as exogenous dangerous invaders. While most studies focused on either the endogenous or the immunological aspect, a number of genes had been implicated in both processes, and this functional overlap was not only found for intracellular components, like cytoskeletal or vesicle traffic factors, but also for receptors, for example CD36, integrins and CD14 (Siamon 2002). Moreover, the diversification of NIM family members with *simu* and *drpr* playing a role in corpse and *nimC1* in bacterial clearance suggests potential similarities in recognition of apoptotic cells and bacteria, leading to the questions: what are the differences between pathogen and apoptotic clearance? How specific are phagocytosis genes for a certain target type? While there are screens published for phagocytosis of pathogens that compare different bacteria, the overlap between these screens is low (**figure 2, p. 20**), and no study has compared apoptotic to bacterial clearance. Cross-specificity of factors has become apparent as a result of testing factors in individual assays for pathogen *or* apoptotic cell phagocytosis, never side-by-side in both, and a systematic analysis of phagocytosis of different food types is missing. We therefore decided to compare phagocytosis of apoptotic cells with phagocytosis of bacteria, and we chose *E. coli* and *S. aureus* as the canonical representatives of Gram-positive and Gram-negative bacteria tested in different previous phagocytosis screens. Additionally we added negatively charged polysterene beads as target particles, because they had been previously used as model particles in phagocytosis studies and screens to examine the engulfment of an, apart from the charge, ligand-free target (Stroschein-Stevenson et al. 2006). For the question of comparing different foods, the phagocytic cell type was of secondary importance. To investigate these two basic questions, finding new glial engulfment factors and comparing phagocytosis of different food types, we collated a list of 86 genes with genes from our glial expression screen, which, based on their similarity to known genes, could play a role in phagocytosis. These genes were selected to find new players in glial phagocytosis. To address the question of specificity, we picked a number of genes not necessarily expressed in glia, but known to be involved in macrophage phagocytosis of a specific food type.

We also added known phagocytosis factors from *Drosophila* and other organisms as controls for the different types of phagocytosis.

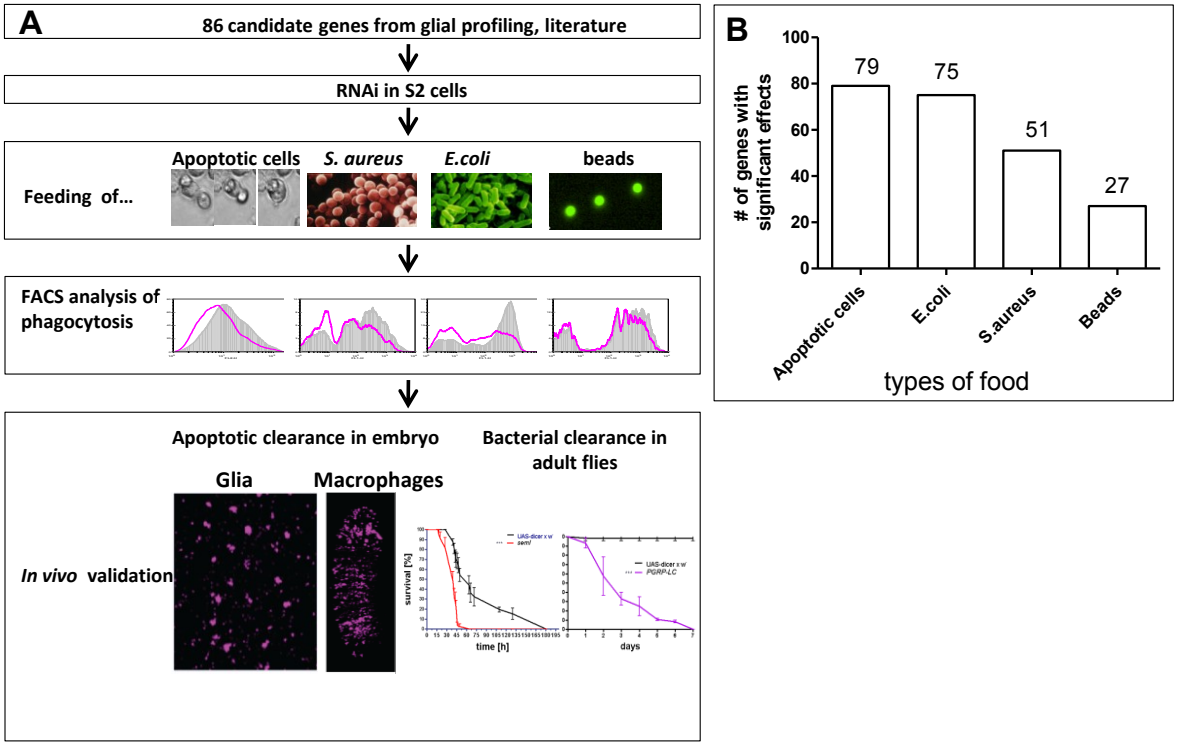


Figure 6: Screen overview.

***A** Workflow of the phagocytosis screen. **B** Distribution of effects found for the different assays. The cut-off for significance was set to effect strengths >10% and concurrence of a q-value from the FDR analysis of <0.05.*

In our selection of genes we were not only interested to find new receptors, but also wanted to examine intracellular factors and their involvement in phagocytosis. Therefore our candidate selection follows a longitudinal axis from secreted and surface factors to more downstream events including signaling, cytoskeletal and vesicle traffic factors and transcription factors. This selection recapitulates the phagocytosis process itself from recognition to engulfment and phagosome maturation (**figure 5, p. 25**).

As we wanted to test these 86 genes in four assays (apoptotic cells, *E. coli*, *S. aureus* and beads), we needed to find experimental conditions allowing to test the candidates on a medium throughput scale. We decided to use the widely employed *Drosophila* S2 cells, a cell line derived from embryonic macrophages. These cells are highly responsive to RNAi treatment allowing for quick and simple generation of

knock downs and screening through a set of candidates; moreover these cells had been used for phagocytosis screens before. Is it sensible to use macrophages to test glial genes? Macrophages and glia share many similarities in corpse clearance, not only functionally, but also molecularly. Many known macrophage phagocytosis factors are upregulated in glia, pointing to these cells having a similar molecular repertoire as macrophages (Alfonso & Jones 2002; Sonnenfeld & Jacobs 1995). Both *simu* and *drpr*, for example, are required in glia as well as macrophages for corpse engulfment, illustrating that glia and macrophages share fundamental mechanisms during phagocytosis (Kurant et al. 2008).

To assess whether a glial gene or another candidate was indeed expressed in macrophages, we used S2 expression profiles from our lab as well as kindly provided by M. Boutros (DKFZ, Heidelberg) and K. Förstermann (Gene Center, Munich). Some of the genes we wanted to test in S2 cells seemed not to be expressed, including members of the PGRP and NIM families (**table 2, p. 29**), but we still included them in the screen because we suspect these expression data to be at least partially false negatives: expression of some of these genes, namely *simu* and *pgrp-lc*, in S2 cells/ macrophages has been published and my in situ hybridizations for *nimB4* and *nimC3* (not shown) reveal that these genes are expressed in embryonic macrophages (Kurant et al. 2008; Rämet et al. 2002). Also, all of these genes are expressed in the embryo according to my RT-PCRs as well as Flybase high throughput expression data (**figure 7, p. 29; table 2, p. 29**) and many of these genes have been tested and found to be expressed in hemocytes at later developmental stages (Royet & Dziarski 2007), suggesting that they are also expressed in macrophages in the embryo.

The primary tissue culture screen was to be followed up by *in vivo* validation, where, depending on a gene's expression, we would use transgenic RNAi to knock down gene function either in glia or macrophages or use available null mutants to test a gene's involvement in phagocytosis. To validate putative new glial phagocytosis factors we looked at glial corpse clearance in the embryonic CNS, while for factors not expressed in glia but putatively involved in apoptotic clearance, we looked at corpse clearance by embryonic macrophages. To assess bacterial clearance by macrophages, we infected adult flies with *E. coli* or *S. aureus* and monitored the flies' survival over time. In this fashion we were able to find new players in glial and macrophage phagocytosis as well as cross-compare the different food types also *in vivo* (**figure 6, p. 27**).

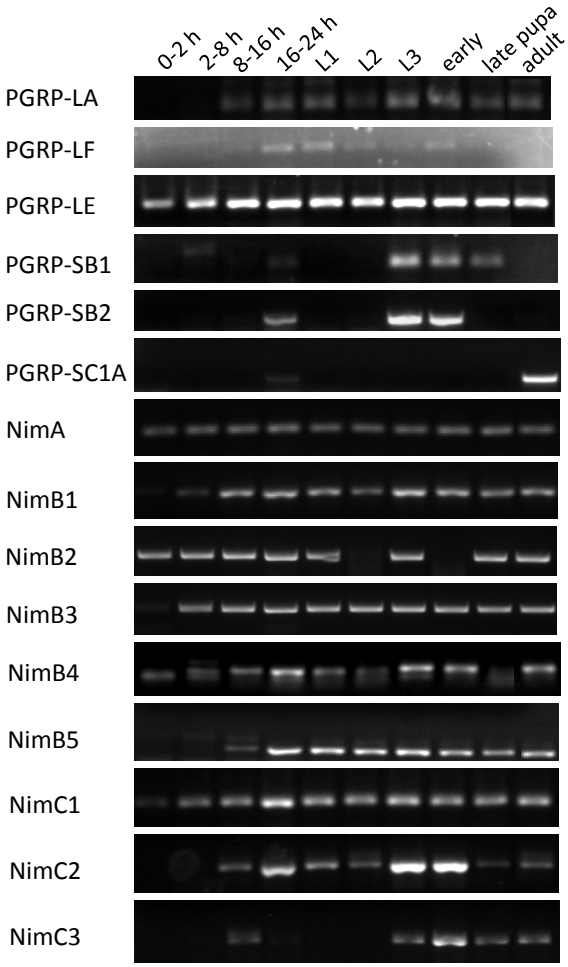


Figure 7: Expression of the NIM and PGRP gene families in development.

Shown are RT-PCRs of extracts from different embryonic and larval stages (L1-3), pupa and adult flies.

Following page:

Table 2: Expression of genes in the screen.

Shown are results from microarray data of embryonic glia (Gaul) and S2 cells (three different profiles from Gaul, Boutros and Foerstermann) as well as high-throughput transcription data from FlyBase. For the NIM and PGRP families additional RT-PCRs have been carried out. simu, pgrp-lc *and* dscam *have been shown to be expressed in S2 cells or embryonic macrophages in the literature.* nimC3 *and* nimB4 *are expressed in embryonic macrophages (*in situs *not shown).*

	Gene name	Glial expression	Embryo: Glia	Embryo: S2/ macrophages: S2 expression Boutros	Embryo: S2/ macrophages: S2 expression Foerstermann	Embryo: S2/ macrophages: S2 expression Fruehauf/Gaul	Embryo: total embryo: Literature or my in situ	Embryo: total embryo: FLyBase/ embryonic expression	Embryo: total embryo: RT-PCR embryo	Adult: Flybase adult expression	Adult: RT-PCR adult	References
NIMs	Draper	↑	+	+	+			+		+		
	CG7447	↑	-	+	+			+		+		
	NimB1		-	-	-			+	+	+	+	
	NimA		-	-	-			+	+	+	+	
	NimC2		-	-	-			+-	+	+-	+	
	NimB4		-	-	-		+	+	+	+	+	
	Simu	↑	-	-	-		+	+		+		Kurant 2008
	NimC1		-	-	-			+	+	+	+	
	NimB5		-	-	-			+	+	+	+	
	NimC3		-	-	-		+	+	+	+	+	
	Hemese		-	-	-			+-		+-		
	NimB2		-	-	-			+	+	+	+	
PGRPs	PGRP-SA		+	+	+			+		+		
	PGRP-SD		-	+	+			+		+		
	PGRP-LC		-	-	-		+	+		+		Ramet 2002
	PGRP-LE		-	-	-			+	+	+	+	
	PGRP-SB1		-	-	-			+	+	+		
	PGRP-LF		-	+	+			+	+	+		
	PGRP-LA		-	+	+			+	+	+	+	
	PGRP-SC1A		-	-	-			+	+	+	+	
	PGRP-SB2		-	-	-			+-	+	+	+-	
Surface proteins	Annexin IX	↑	+	+	+			+		+		
	EGFR	↔	+	+	+			+		+		
	Croquemort-like	↑	+	-	-			+		+		
	PSR		-	+	+			+		+		
	Calreticulin	↑	+	+	+			+		+		
	Croquemort		+	+	+			+		+		
	CG10702	↑	-	-	-			+		+		
	Mdr65	↑	-	-	-			+		+		
	LDL-R	↑	-	-	+			+		+		
	W	↑	+	+	+			+		+		
	Eiger	↑	+	+	+			+		+		
Bacterial recognition	Dscam		-	+	+	+		+		+		Watson 2005
	TEP4	↔	+	+	+			+		+		
	TEP2		+	+	+			+		+		
	TEP1		-	-	-			+-		+		
	Mcr	↔	+	+	+			+		+		
	dSR-CI		+	+	+			+		+		
ECM	MMP-1	↑	+	+	+			+		+		
	Hemomucin	↑	+	+	+			+		+		
	myospheroid	↔	+	+	+			+		+		
	TIMP	↑	-	+	+			+		+		

	Gene name	Glial expression	Embryo: Glia	Embryo: S2/ macrophages: S2 expression Boutros	Embryo: S2/ macrophages: S2 expression Foerstermann	Embryo: S2/ macrophages: S2 expression Fruehauf/Gaul	Embryo: tot. em.: FLyBase/ embryonic expression	Adult: Flybase adult expression
ECM	Dystroglycan	↑	+	+	+	+	+	+
	Gliolectin	↔	+	+	+	+	+	+
	inflated	↔	+	+	+	+	+	+
	Malvolio	↔	+	+	+	+	+	+
non RTK	Shark	↔	+	+	+	+	+	+
	Fps85D	↑	+	+	+	+	+	+
	Pvr	↑	+	+	+	+	+	+
JNK	Jun-related antigen	↔	+	+	+	+	+	+
	Puckered	↔	+	+	+	+	+	+
PIP metabolism	Pi3K68D	↔	+	+	+	+	+	+
	Pi3K59F	↔	-	+	+	+	+	+
	Pi3K92E	↔	+	+	+	+	+	+
	Skittles	↑	+	+	+	+	+	+
	Sac1	↔	+	+	+	+	+	+
	PTEN		+	+	+	+	+	+
small GTPases	Rac2	↑	+	+	+	+	+	+
	Vav	↑	+	+	+	+	+	+
	Rac1	↔	+	+	+	+	+	+
	Rho1	↔	+	+	+	+	+	+
	Cdc42	↔	+	+	+	+	+	+
	Mig-2-like	↔	+	+	+	+	+	+
	Trio		+	+	+	+	+	+
Cytoskeleton	Myoblast City	↑	+	+	+	+	+	+
	Arp66B	↔	+	+	+	+	+	+
	Hem/ Kette	↔	+	+	+	+	+	+
	dCED-12	↔	+	+	+	+	+	+
	dCED-6	↔	+	+	+	+	+	+
	SCAR	↔	+	+	+	+	+	+
	Short stop	↑	+	+	+	+	+	+
	SRA-1	↔	+	+	+	+	+	+
	Crk	↑	+	+	+	+	+	+
	Dmoesin	↑	+	+	+	+	+	+
	Coracle	↑	+	+	+	+	+	+
Vesicle traffic	zetaCOP	↑	+	+	+	+	+	+
	Gartenzwerg)	↔	+	+	+	+	+	+
	bCOP	↔	+	+	+	+	+	+
	Vha14	↑	+	+	+	+	+	+
	α-Adaptin	↑	+	+	+	+	+	+
	Shibire	↔	+	+	+	+	+	+
	Cysteine proteinase-1	↑	+	+	+	+	+	+
	Cyp4g15	↑	-	-	-	-	+	+
	Syb	↑	+	+	+	+	+	+
EcR	Eip63E	↔	+	+	+	+	+	+
	EcR	↑	-	+	+	+	+	+

2 Cell-based screen - methodology

2.1 First and secondary RNAi design

As we set out to conduct a medium throughput candidate screen for phagocytosis factors, we decided to employ the relatively fast RNAi technology to generate gene knockdowns. Michael Boutros kindly provided us with the dsRNA library for our 86 candidate genes. The RNAs provided belong to the second generation dsRNA library (Heidelberg 2). In contrast to the first generation library, the design of these new probes had been optimized to minimize off-target-effects (OTE). This has been achieved by computational tools (NEXT-RNAi, See Material and Methods 8.5, 8.6) that excluded probes with ubiquitous trinucleotide tandem repeats (CAN repeats), 19 nucleotide siRNA matches in other genes, regions of low complexity that are known to generate unspecific OTE and short interfering RNAs (siRNAs) containing microRNA seeds (Horn et al. 2010).

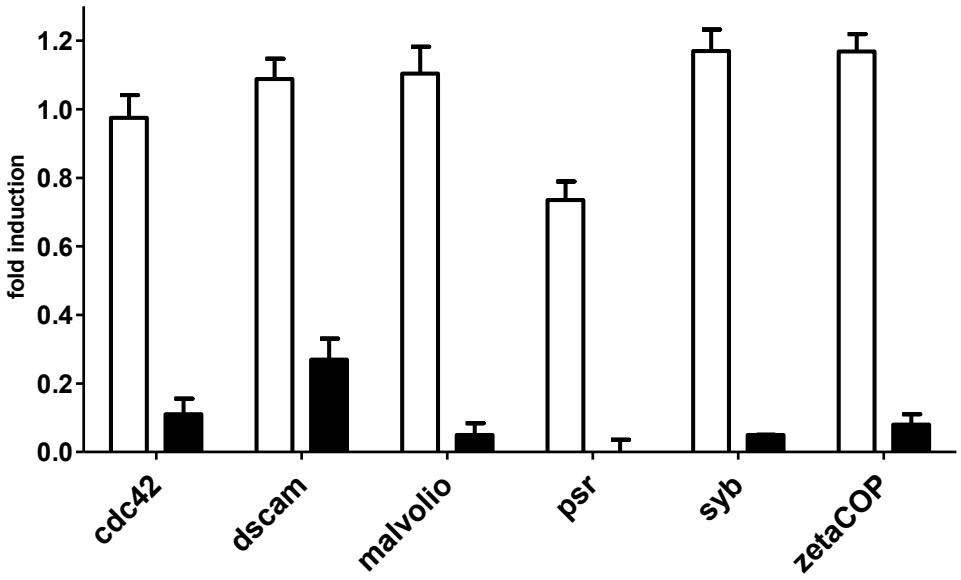


Figure 8: qRT-PCR of RNAi in S2 cells.

RNAi efficiency was tested using qRT- PCR. Bars represent fold changes of expression in comparison to untreated cells. White bars show fold changes of the indicated genes in control GFP RNAi treated cells, black bars in the target RNAi. RNAi leads to gene knock downs from ~60% – 99%.

Despite the optimization of probe design, computational analyses of this library still predict 26.6% of probes to have siRNA hits in at least one other gene and 0.5% have CAN repeats. Because of the imperfection of RNAi with potential false positives due to off-target effects and false negatives due to insufficient knockdowns, secondary testing of RNAi results has become the rule. Therefore, we had to evaluate the efficiency and specificity of RNAi in our screen. We incubated the cells with the dsRNA for three days. To test knock down efficiencies we performed quantitative real-time PCR (qRT-PCR) analyses and observed RNA reductions of 60-99%, which is in line with what should be expected (Thomas Horn et al. 2010, **figure 8, p. 31**).

To make sure that the observed effects are specific, for 19% of our candidates we designed secondary RNAs targeting a non-overlapping region of the tested gene, creating two independent probes (see 8.5 in Materials and Methods). Effects deviating more than 10% from each other, which corresponds to the experimental variability, or cases where one result was above and the other below our effect threshold of 10% were considered discrepancies between the first and second probe. We observed discrepant effects in only 18.75% of the cases, indicating that our findings were mostly attributable to specific gene knock downs (**table 3, p. 33**). How does this amount of validation and our level of OTE compare to other studies? Before 2006/2007, when a number of papers pointed out the extent of OTE in RNAi screens, secondary RNAs were not common for validating results from first round RNAi screens. Hits from the first screen were validated by repeating the initial RNAi experiment with newly synthesized probes of the same sequence and a different functional assay, for example a different reporter construct (Boutros et al. 2004; Muller et al. 2005; Gesellchen et al. 2005; Gwack et al. 2006; Eulalio et al. 2007). From 2008 on it became more common to validate hits from genome-wide RNAi screens using secondary non-overlapping dsRNAs. The number of discrepant results in these screens varies greatly from no discrepancies in one study (Chittaranjan et al. 2009, 20 genes retested), 15-20% discrepancies (Chew et al. 2009; T. Liu et al. 2009; Wendler et al. 2010, 22-75 genes retested), ca. 40 % discrepancies (Hao et al. 2008; Sathyanarayanan et al. 2008, 176/ 286 genes retested) to 84% discrepancies (Guo et al. 2008, 847 genes retested) and is therefore somewhat inversely proportional to the number of retested genes. Our rate of 18.75% discrepancy for 19 retested genes is therefore in the range of what we would expect from the literature.

Table 3: Secondary RNA effects are mostly consistent with primary RNAs'.

	Effects of genes on phagocytosis				Difference between first and secondary RNA			
Gene name	Apoptotic	E. coli	S. aureus	Beads	Apoptotic	E. coli	S. aureus	Beads
Draper	-46	-14	-15	-6				
Draper_2ndary	-39	-14	-11	1	7.2	0.7	4.1	5.0
NimA_1	-19	-9	-9	-7				
NimA_2ndary	-13	-7	-8	-6	5.2	2.6	1.4	0.6
Simu	-16	-19	-11	-5				
Simu_2ndary	-15	-16	-20	-7	0.7	2.4	8.8	2.5
PGRP-LC	-14	-38	-17	-7				
PGRP-LC_2ndary	-19	-29	-14	-15	5.4	9.1	2.3	7.8
Megalin	-13	-14	-8	-4				
Megalin_2ndary	-10	-11	-7	3	2.6	2.3	0.8	0.9
Mdr65	-10	-16	-10	-8				
Mdr65_2ndary	-12	-9	-5	-32	1.4	7.2	4.1	23.7
Annexin IX	-19	-23	-9	-7				
AnnIX_2ndary	-13	-15	-3	-9	6.5	8.8	5.6	1.9
Mcr	-13	-16	-14	-4				
Mcr_2ndary	-14	-14	-14	-21	1.0	2.0	0.4	17.0
TEP2	-13	-10	-1	-4				
TEP2_2ndary	-11	-9	-2	-13	1.9	0.6	0.4	9.5
DSCAM	-16	-22	-14	-5				
DSCAM_2ndary	-11	-21	-15	2	5.3	1.3	0.7	2.9
MMP-1	-13	-12	-6	-8				
MMP-1_2ndary	-18	-19	-8	-3	5.7	6.6	2.1	5.2
Gliolectin	-12	-19	-7	-11				
Glec_2ndary	-3	-19	-3	-4	9.7	0.3	4.0	6.6
Puckered	-2	-28	-21	0				
Puc_2ndary	0	-23	-13	-1	1.9	5.4	7.9	1.3
Pi3K68D	-23	-25	-18	-10				
PI3K68D_2ndary	-13	-33	-25	-1	10.0	8.1	6.3	9.2
Rac2	-22	-22	-9	-7				
Rac2_2ndary	-26	-23	-6	2	3.3	1.2	3.1	4.8
Cdc42	-11	-13	4	-9				
Cdc42_2ndary	-42	-18	-12	-13	31.0	4.7	15.3	3.5
average difference between probes					6.2	4.0	4.2	6.4

To assess OTE of RNAi, secondary non-overlapping RNAs were designed against 19% (16 genes) of the candidate genes. Shown are effects of both RNAs for all four assays, the difference between results and the result of the t-test between probes to assess significance. Effect deviation >10%, which corresponds to the experimental variability, and/ or across our effect threshold of 10% was considered to be a discrepancy. Discrepancies were observed in 12 cases or 19% of the genes.

2.2 Phagocytosis assay and flow cytometry analysis

We wanted to measure how different RNAi treatments affect the extent to which the cells could eat different types of food, so we fed them different types of particles: stained apoptotic S2 cells, AlexaFluor488-labeled *E. coli* and *S. aureus* and carboxylated, negatively charged AlexaFluor488-labeled beads (all three commercially available, see 8.2 in Material and Methods).

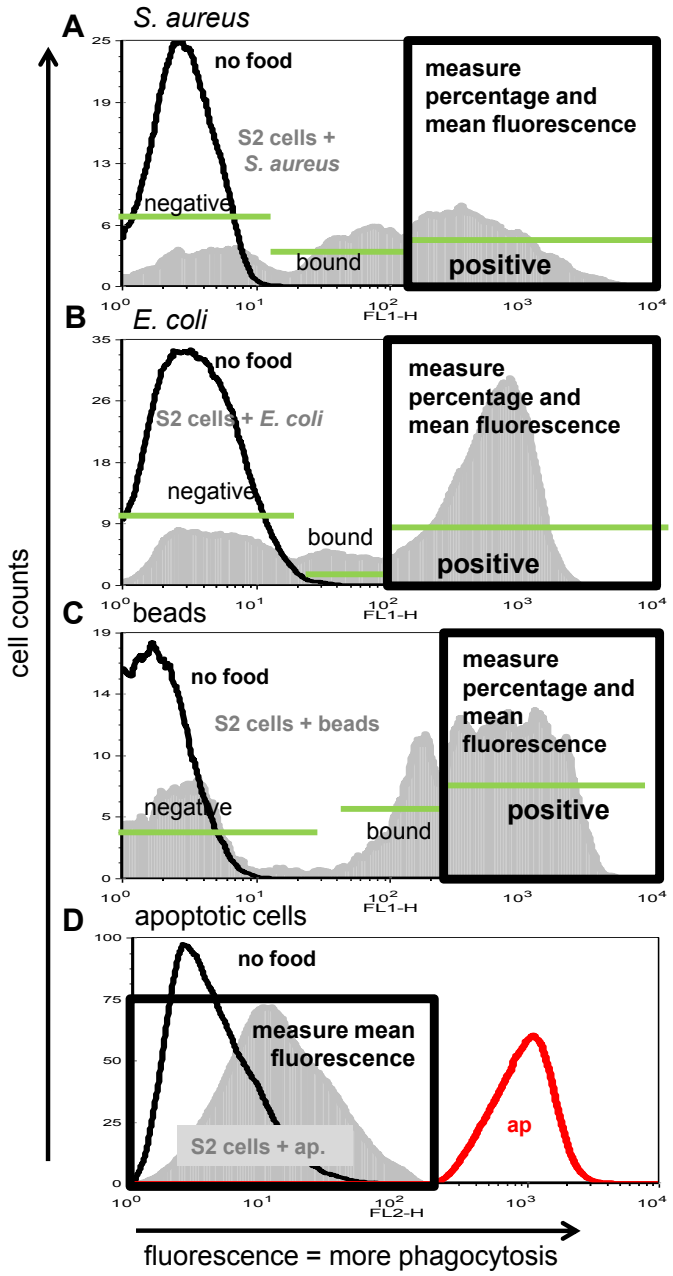


Figure 9: Analysis of phagocytosis using flow cytometry.

Shown are histograms of cell fluorescence distributions. In each plot, the black line indicates S2 cells before eating, and the gray filled area the distribution after phagocytosis of fluorescent food, therefore the population is shifted to the right. ***A-C*** *Due to quenching of extracellular fluorescence, cells with particles only stuck to them, but not inside of them can be distinguished, therefore yielding three populations of cells: 'negative' without particles, 'bound' with particles on the surface, and 'positive' with particles inside the cell. To evaluate, how many cells ate and how much they ate, the percentage and mean fluorescence of the cells with engulfed particles ('positive') are determined.* ***D*** *Apoptotic cell assay. The eaters alone are shown in black. The red fluorescent apoptotic cells alone are shown in red. The grey filled area indicates the distribution of eaters after incubation with apoptotic cells. The population is shifted to the right. The mean red fluorescence of the eating population is determined in order to evaluate, how many apoptotic cells the S2 cells phagocytosed.*

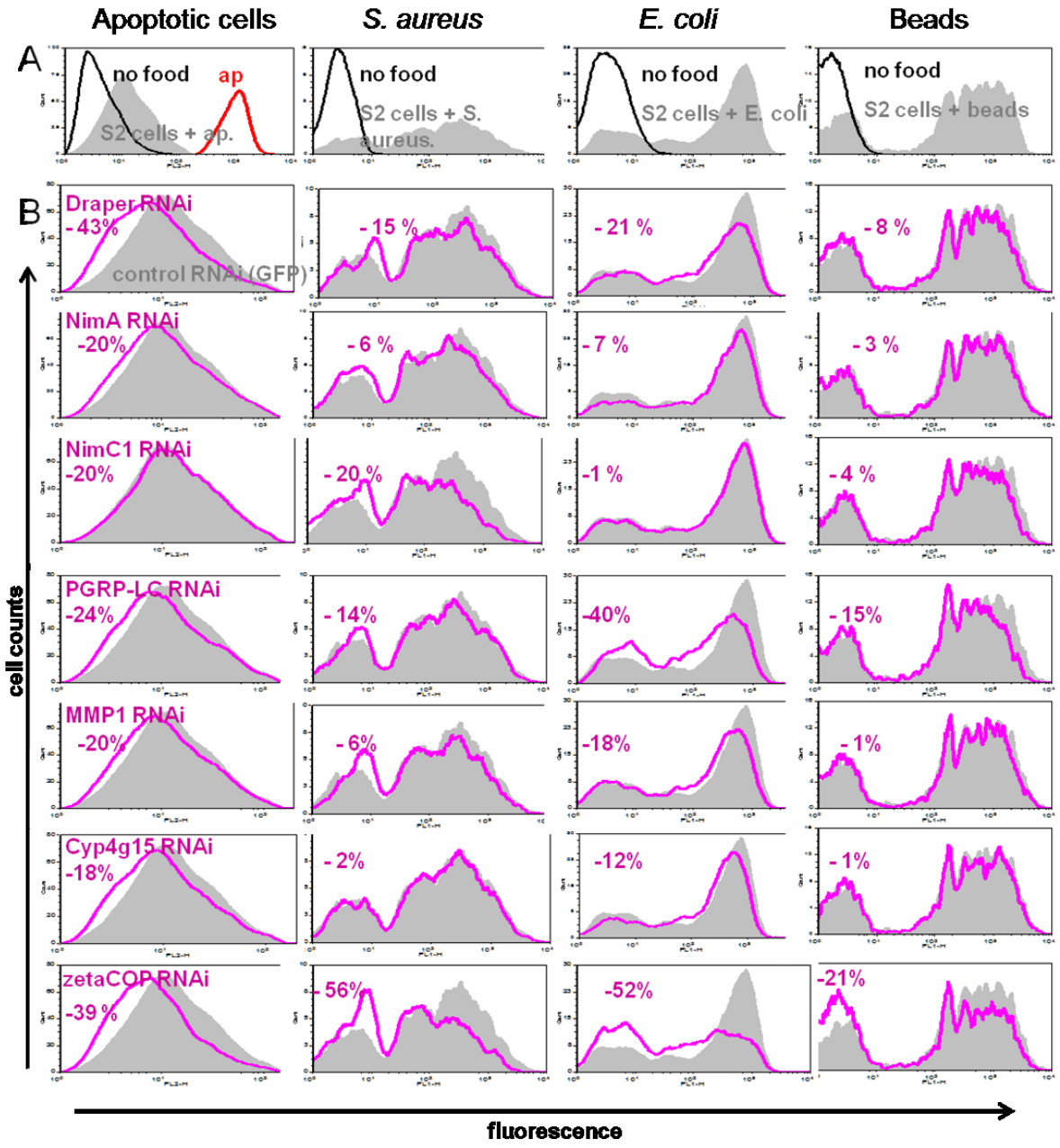


Figure 10: Flow cytometry phagocytosis screen - examples.

Unstained cells are incubated with fluorescent 'food'. Depicted are fluorescence (=food) distributions in the eater cells. ***A****. Black, cells without food and grey, after eating. The red line in the apoptotic assay shows the red stained apoptotic cells alone. Peaks in the beads assay represent individual ingested beads.* ***B****. Effects of RNAi. In grey: cells treated with control RNA (GFP); magenta, with candidate RNA. Decrease of peak height and a shift to the left represent a reduction of phagocytosis. Indicated percentages are normalized effects on phagocytosis as compared to GFP RNAi-treated cells. Each assay was performed in replicates and repeated 2-7 times, shown is a representative example.*

Different techniques have been used to score phagocytosis events, namely manual and automated microscopical evaluation and flow cytometry (Cronin et al. 2009; Stroschein-Stevenson et al. 2009; Rämet et al. 2002; Philips et al. 2005). Notably, these screens produced only small overlap among each other, which is probably due to the different techniques employed (**figure 2, p. 20; table 1, p. 18**). As we wanted a fast, quantitative readout for our first round of screening, we decided to use flow cytometry for our analyses (8.3 in Material and Methods). Here, we were able to analyze thousands of cells for ingested particles each second. The method works as follows: the more cells eat fluorescent particles, the more fluorescent they become themselves (**figure 9, p. 34**). The fluorescence for each cell is measured, and in total 5000 cells are evaluated in that fashion. For the fluorescently labeled beads and dead bacteria, quenching of extracellular fluorescence is possible, which means that particles outside the eating cells or particles stuck to the eating cells' surface can be excluded from the analysis. Here, only the fluorescence of the eaters with engulfed beads or bacteria are evaluated for the amount of engulfed particles. However, the 'living' apoptotic cells cannot be quenched, because they would ingest the quencher through endocytosis eliminating their fluorescence. An additional complication is that apoptotic cells do not form a uniform particle population like bacteria or beads, but display a range of sizes from early apoptotic cells, which have not undergone shrinking yet, to shrunken apoptotic cells and finally apoptotic blebs. This heterogeneity of the apoptotic cell population leads to a shift in the fluorescence of the eater cell population rather than the formation of distinct peaks of negative and positive cell populations, as occurs with beads and bacteria (**figure 9**). Therefore, for the apoptotic assay, we determined that the analysis yielding the best differential between negative (*gfp*) and positive control (*zCOP*) to evaluate the whole population of eating cells for the amount of ingested apoptotic cells. For example, RNAi of the known phagocytosis gene *zCOP* reduces the cells' capacity to engulf red fluorescent apoptotic cells, resulting in lower fluorescence of the eater cell population compared to the control (*gfp* RNAi) treated cells. This corresponds to a reduction in phagocytosis of ~40 % (**figure 10, p. 36**). Also, we optimized eating times and the amounts of food for all assays to achieve a maximal differential between positive and negative control.

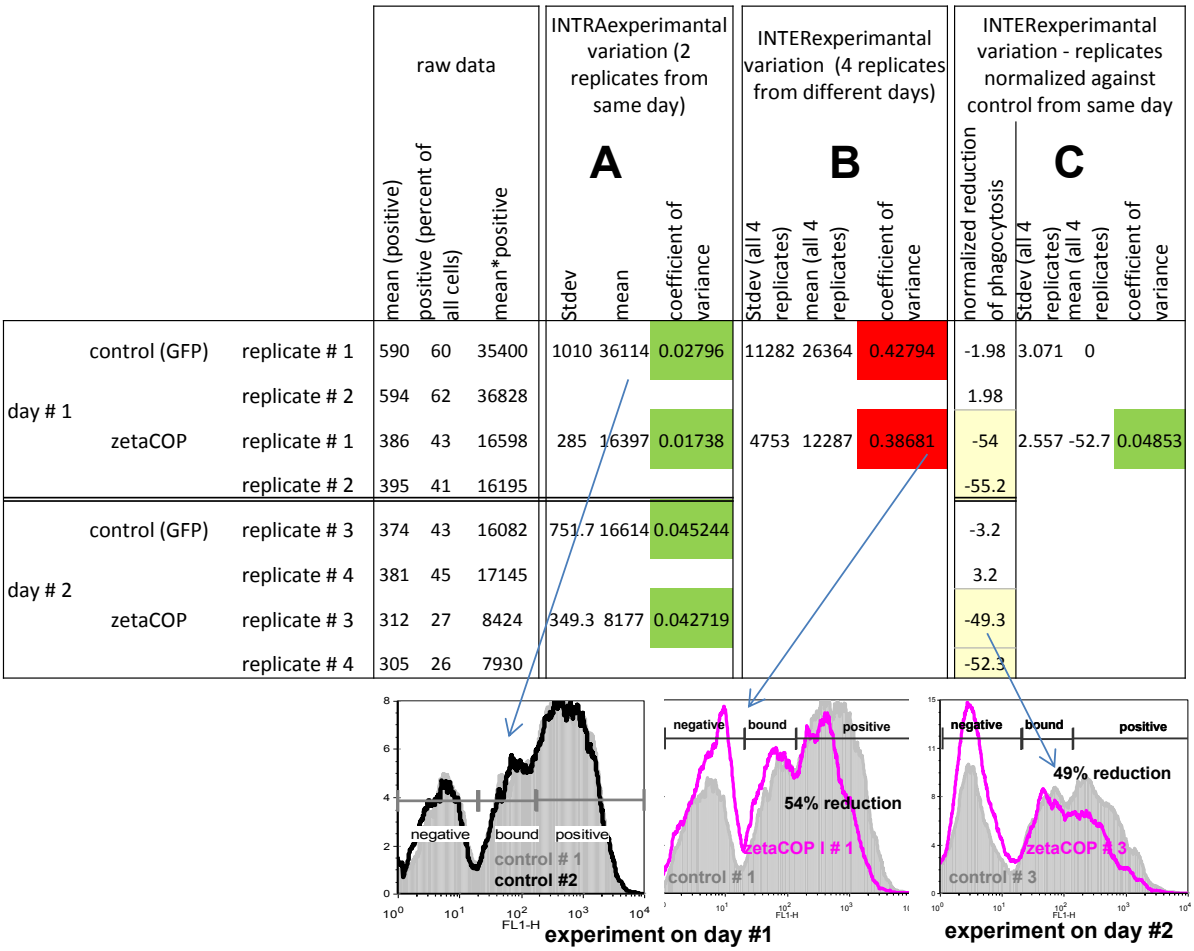


Figure 11: Normalization decreases interexperimental variation.

Shown are flow cytometry analyses of S. aureus *phagocytosis as an example.* ***A*** *Intraexperimental variation: duplicates on each plate have the average standard deviation of 3%.* ***B*** *raw data variation between same genotypes on different days is much higher than between different genotypes on same day requiring normalization. C Normalization allows comparison of experiments performed on different days. Average standard deviation between experiments performed on different days: 10%.*

2.3 Normalization and quality control

To rule out indirect effects on phagocytosis by RNAi affecting cell viability, we determined the size of the cells as a read out to analyze cell apoptosis using the forward scatter parameter of the flow cytometer (**figure 12, p.40**). Apoptotic cells are on average 63% smaller than healthy cells. None of the RNAi treatments induced apoptosis, showing that a potential reduction of phagocytosis is not a secondary consequence of cell death. Because flow cytometry is a highly sensitive method, variations in cell states, cell media, apoptotic cell preparations and handling lead to experimental variability (**figure 11, p.38**). To be able to compare different experiments, we normalized each experiment against a negative control (*gfp* RNAi) yielding an average standard deviation between experiments of 10% and standard error of the mean of 3.5 % (**figure 13, p.41**). We repeated assays 3-7 times on different days and confirmed overall assay performance for each experiment by including a positive control (*zCOP*), which was expected to have an effect on phagocytosis greater than 20%.

2.4 Effect strength

RNAi does not completely remove gene function, but only reduces it. Depending on a gene's requirement in the cell and the longevity of the protein, this may or may not lead to a phenocritical reduction in gene activity. Moreover, phagocytosis is a highly redundant process, where the lack of individual genes can be compensated by other genes. The worm, even though only 131 cells die during all of development, safeguarded their phagocytic removal by two independent signaling cassettes, but not even knockout of both of them completely abolishes phagocytosis (Reddien & Horvitz 2004). The strongest phenotypes observed in gene knockouts in worm, fly and mouse only lead to a two to three-fold increase of apoptotic material *in vivo*, which required us to adjust our expectations for effect strengths, especially since we were not creating nulls, but gene knockdowns of presumably varying extent. Consequently, although we optimized RNAi treatment protocols, eating durations and eater-to-food-ratios, the demonstrated effect strengths are moderate to weak (**figure 13, p. 41**).

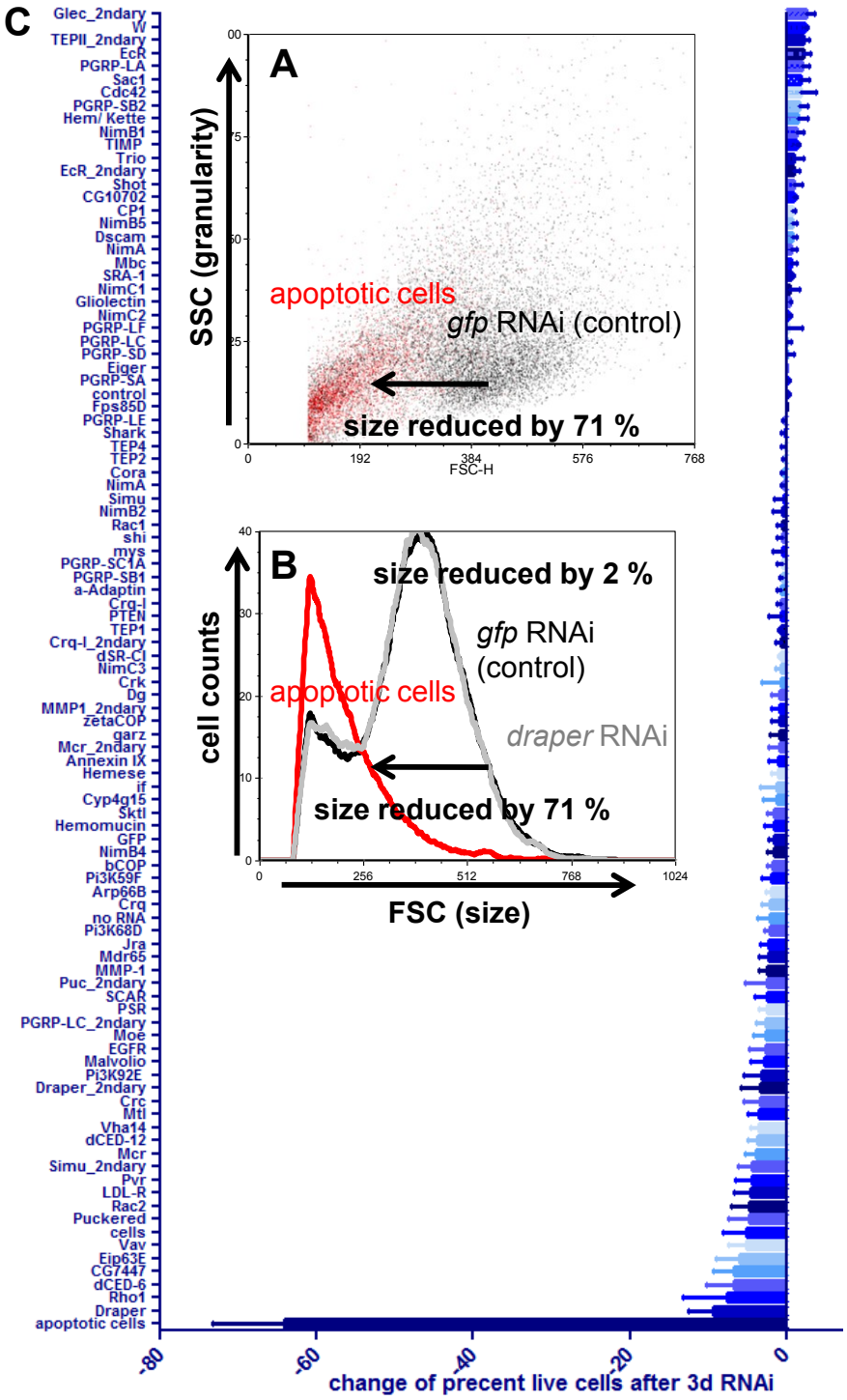


Figure 12: RNAi of candidate genes does not affect cell viability.

A Dot plot showing forward (size) and side scatter (granularity) of cells treated with RNA against GFP (control, black) and with etoposide for 16 h to induce apoptosis (red). Apoptotic cells are 71% smaller than live cells. **B** Histogram of live cells (black), apoptotic cells (red), and cells treated with draper RNAi for 3d, the gene with the strongest overall effect on cell viability (on average 9 %, in this example 2 %). **C**

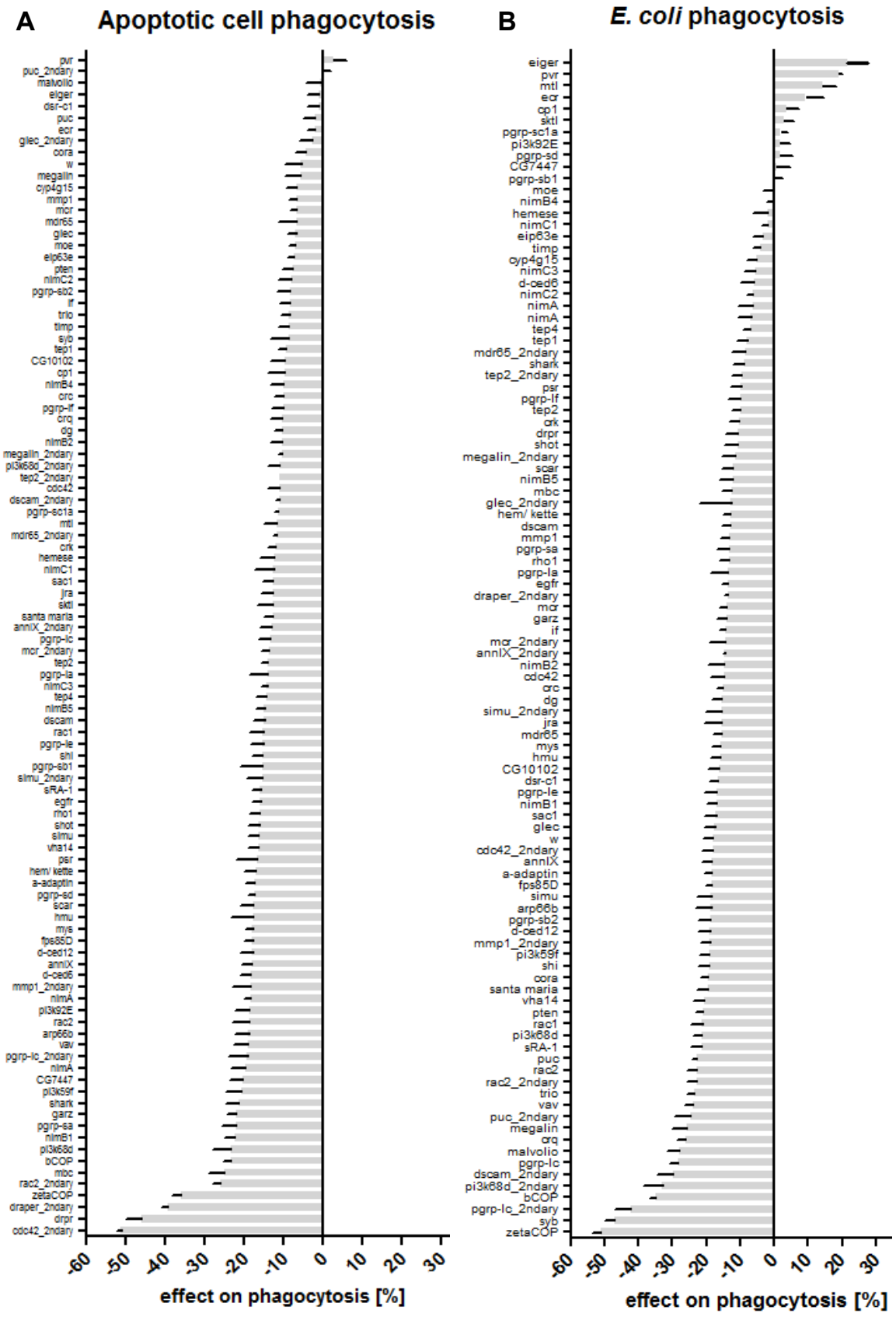
Changes in the amount of live cells after 3 days of RNAi treatment against specific *candidate genes.*

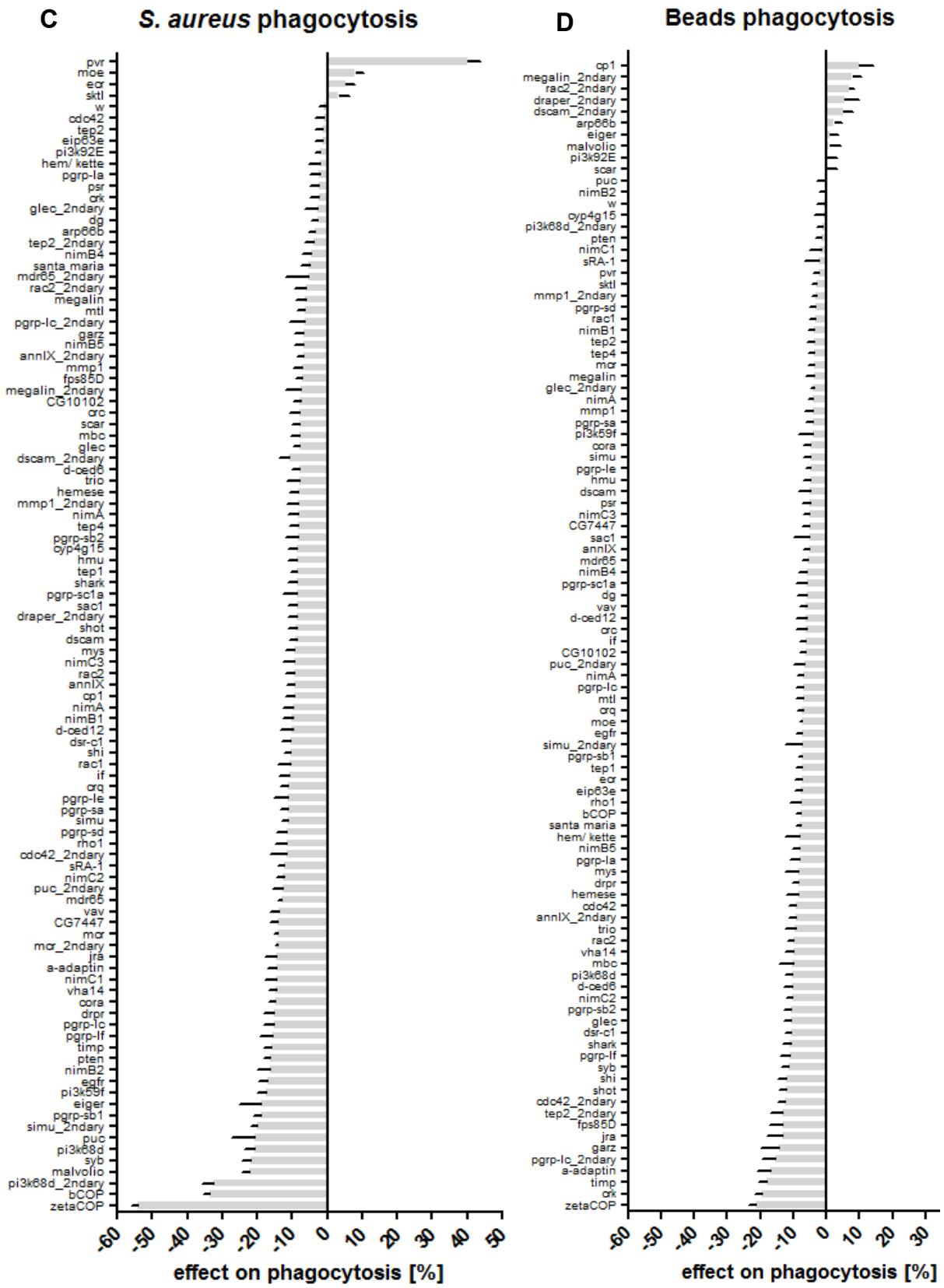
The genes with the strongest effects led to a reduction in phagocytosis of roughly two-fold for apoptotic cells and bacteria (46% *drpr* for apoptotic cells, 52% *zCOP* for *E. coli,* 54% *zCOP* for *S. aureus)* and 21% for beads (*zCOP*). The average effects for significant hits ranged from a 14-18% reduction of phagocytosis. However our results were highly reproducible, leading us to use a relatively low cut-off of 10% change in phagocytosis and concurrent q-values from the false discovery rate (FDR) analysis of <0.05. FDR analyses of statistical significance showed significant values for 79 (apoptotic cell assay), 75 (*S. aureus* assay), 51 (*E. coli* assay) and 27 (beads assay) different genes (**figure 6B, p. 27**). We found the most genes for apoptotic cell phagocytosis. This is not due to an internal bias in the candidate selection, because even though we selected a number of genes based on their homology to known apoptotic cell receptors, we also included many known bacterial receptors, and the majority of candidates were not clearly attributable to apoptotic or bacterial clearance (**figures 5, 14, pages 25, 45**). The effects found were largely significant and therefore provided a good starting point for further secondary screening and *in vivo* validation.

Following pages:

Figure 13: Effects of RNAi knock downs of candidate genes on phagocytosis of different target particles.

*Phagocytosis of **A** apoptotic cells, **B** E. coli, **C** S. aureus and **D** beads. Bar charts represent the mean and the SEM of 5-15 individual experiments for each gene. Results are sorted by effect strength.*


A
Apoptotic cell phagocytosis
pvr
puc_2ndary
malvolio
eiger
dsr-c1
puc
ecr
glec_2ndary
cora
w
megalin
cyp4g15
mmp1
mcr
mdr65
glec
moe
eip63e
pten
nimC2
pgrp-sb2
if
trio
timp
syb
tep1
CG10102
cp1
nimB4
crc
pgrp-lf
crq
dg
nimB2
megalin_2ndary
pi3k68d_2ndary
tep2_2ndary
cdc42
dscam_2ndary
pgrp-sc1a
mtl
mdr65_2ndary
crk
hemese
nimC1
sac1
jra
sktl
santa maria
annIX_2ndary
pgrp-lc
mcr_2ndary
tep2
pgrp-la
nimC3
tep4
nimB5
dscam
rac1
pgrp-le
shi
pgrp-sb1
simu_2ndary
sRA-1
egfr
rho1
shot
simu
vha14
psr
hem/ kette
a-adaptin
pgrp-sd
scar
hmu
mys
fps85D
d-ced12
annIX
d-ced6
mmp1_2ndary
nimA
pi3k92E
rac2
arp66b
vav
pgrp-lc_2ndary
nimA
CG7447
pi3k59f
shark
garz
pgrp-sa
nimB1
pi3k68d
bCOP
mbc
rac2_2ndary
zetaCOP
draper_2ndary
drpr
cdc42_2ndary
-60
-50
-40
-30
-20
-10
0
10
20
30
effect on phagocytosis [%]
B
E. coli phagocytosis
eiger
pvr
mtl
ecr
cp1
sktl
pgrp-sc1a
pi3k92E
pgrp-sd
CG7447
pgrp-sb1
moe
nimB4
hemese
nimC1
eip63e
timp
cyp4g15
nimC3
d-ced6
nimC2
nimA
nimA
tep4
tep1
mdr65_2ndary
shark
tep2_2ndary
psr
pgrp-lf
tep2
crk
drpr
shot
megalin_2ndary
scar
nimB5
mbc
glec_2ndary
hem/ kette
dscam
mmp1
pgrp-sa
rho1
pgrp-la
egfr
draper_2ndary
mcr
garz
if
mcr_2ndary
annIX_2ndary
nimB2
cdc42
crc
dg
simu_2ndary
jra
mdr65
mys
hmu
CG10102
dsr-c1
pgrp-le
nimB1
sac1
glec
w
cdc42_2ndary
annIX
a-adaptin
fps85D
simu
arp66b
pgrp-sb2
d-ced12
mmp1_2ndary
pi3k59f
shi
cora
santa maria
vha14
pten
rac1
pi3k68d
sRA-1
puc
rac2
rac2_2ndary
trio
vav
puc_2ndary
megalin
crq
malvolio
pgrp-lc
dscam_2ndary
pi3k68d_2ndary
bCOP
pgrp-lc_2ndary
syb
zetaCOP
-60
-50
-40
-30
-20
-10
0
10
20
30
effect on phagocytosis [%]


C
S. aureus phagocytosis
pvr
moe
ecr
sktl
w
cdc42
tep2
eip63e
pi3k92E
hem/ kette
pgrp-la
psr
ork
glec_2ndary
dg
arp66b
tep2_2ndary
nimB4
santa maria
mdr65_2ndary
rac2_2ndary
megalin
mtl
pgrp-lc_2ndary
garz
nimB5
annIX_2ndary
mmp1
fps85D
megalin_2ndary
CG10102
orc
scar
mbc
glec
dscam_2ndary
d-oed6
trio
hemese
mmp1_2ndary
nimA
tep4
pgrp-sb2
cyp4g15
hmu
tep1
shark
pgrp-sc1a
sac1
draper_2ndary
shot
dscam
mys
nimC3
rac2
annIX
cp1
nimA
nimB1
d-oed12
dsr-c1
shi
rac1
if
crq
pgrp-le
pgrp-sa
simu
pgrp-sd
rho1
cdc42_2ndary
sRA-1
nimC2
puc_2ndary
mdr65
vav
CG7447
mor
mor_2ndary
jra
a-adaptin
nimC1
vha14
cora
drpr
pgrp-lc
pgrp-lf
timp
pten
nimB2
egfr
pi3k59f
eiger
pgrp-sb1
simu_2ndary
puc
pi3k68d
syb
malvolio
pi3k68d_2ndary
bCOP
zetaCOP
-60
-50
-40
-30
-20
-10
0
10
20
30
40
50
effect on phagocytosis [%]
D
Beads phagocytosis
cp1
megalin_2ndary
rac2_2ndary
draper_2ndary
dscam_2ndary
arp66b
eiger
malvolio
pi3k92E
scar
puc
nimB2
w
cyp4g15
pi3k68d_2ndary
pten
nimC1
sRA-1
pvr
sktl
mmp1_2ndary
pgrp-sd
rac1
nimB1
tep2
tep4
mor
megalin
glec_2ndary
nimA
mmp1
pgrp-sa
pi3k59f
cora
simu
pgrp-le
hmu
dscam
psr
nimC3
CG7447
sac1
annIX
mdr65
nimB4
pgrp-sc1a
dg
vav
d-oed12
orc
if
CG10102
puc_2ndary
nimA
pgrp-lc
mtl
crq
moe
egfr
simu_2ndary
pgrp-sb1
tep1
ecr
eip63e
rho1
bCOP
santa maria
hem/ kette
nimB5
pgrp-la
mys
drpr
hemese
cdc42
annIX_2ndary
trio
rac2
vha14
mbc
pi3k68d
d-oed6
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shot
cdc42_2ndary
tep2_2ndary
fps85D
jra
garz
pgrp-lc_2ndary
a-adaptin
timp
ork
zetaCOP
-60
-50
-40
-30
-20
-10
0
10
20
30
effect on phagocytosis [%]

3 Validation of screen results

We wanted to find new candidates involved not only in the phagocytosis of apoptotic cells, but also of bacteria and beads. To assess and correctly ascertain the validity of screen results, correct statistical procedures are necessary to estimate the number of false positive hits (FDR analysis, Benjamini & Hochberg 1995, **figure 14, p. 45**). To evaluate the quality of our screen results, we applied three commonly used measures: firstly, secondary non-overlapping RNAs for 19% of the candidate genes were designed and tested, which produced results mostly congruent with our primary probes (19% discrepancy, **table 3, p. 33**). Secondly, our results were subjected to comparison with other S2 cell phagocytosis screens in the literature. In a next step we conducted genetic *in vivo* analyses, where we tested whether an identified gene plays a role in a given process – not only under the isolated cell culture conditions, but also in the complex milieu of a whole organism.

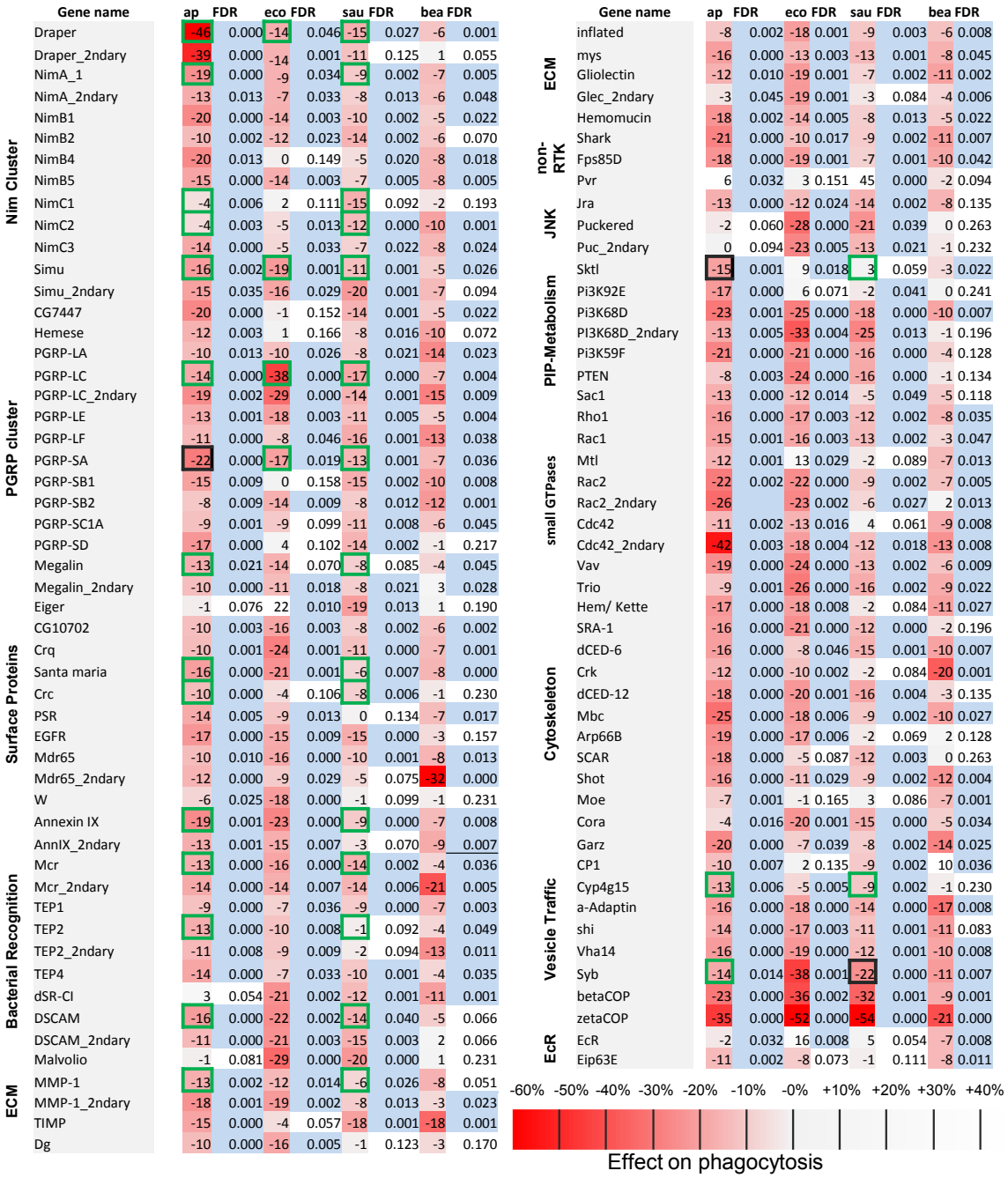


Figure 14: Results and q-values from the FDR analysis of the cell based phagocytosis screen.

For each food type, the mean of 3-7 independent experiments is shown and the effect strength is indicated by a heatmap. The second column for each assay shows q-values for the results. Significant results are indicated in blue (P<0.05), non-significant in white (P>0.05). Boxes around tested genes represent in vivo *testing: green boxes indicate a confirmatory* in vivo *result, black boxes a discrepant one. ap, apoptotic cells; eco,* E. coli*; sau,* S. aureus*; bea, beads.*

3.1 *In vivo* validation – candidates and methodology

The recent generation of a genome-wide transgenic RNAi library in *Drosophila* now allows for relatively simple and fast conditional gene inactivation *in vivo*. Recent screens used this new technology and combined tissue culture RNAi with *in vivo* RNAi, thereby trying to confirm cell culture results in the whole organism, and we decided to use this combined approach for our screen as well (Saj et al. 2010; Port et al. 2011). Our cell-based screen systematically compared eating of different types of food including apoptotic cells for the first time. We were interested in learning whether the candidates yielded from the screen are also relevant players *in vivo* and therefore proceeded to genetic testing in the whole fly. We selected 18 genes that seemed the most interesting to us, and took genes from different cellular compartments (**figure 21A, p. 84**). Most of the genes picked had been described in other functional contexts, but never implicated in phagocytosis before. Five genes were new and had never been implicated in any functional process. Like the initial selection, the genes we picked for *in vivo* testing were intersected from different lines of interest: first and foremost we chose genes highly expressed in glia, because investigating their function was our main motivation for the entire endeavor. However we also included promising candidates based on the fact that they had not been functionally described yet or had shown unexpected cress-specificity in the cell-based screen.

The cell-based screen provided us with a list of interesting candidates potentially involved in phagocytosis of apoptotic cells and bacteria. As we wanted to specifically find new factors for apoptotic clearance in the developing nervous system, we proceeded to test the most interesting candidates *in vivo*, using the *Drosophila* embryo as a model system. We were very surprised to find in our cell-based screen that many of the tested genes were not required exclusively for one type of phagocytosis, in particular apoptotic cell receptors, but also for engulfment of at least one type of tested bacteria. Additionally, we found some factors that had never before been implicated in phagocytosis of bacteria. To see whether these cell-based findings track *in vivo*, we tested whether adult flies lacking any of these factors become more susceptible to bacterial infection. Consequently, because of the amount of cross-specificity we found in our screen, we tested all 18 genes in at least two *in vivo* assays: apoptotic clearance in the *Drosophila* embryo, either in glia or in macrophages and bacterial clearance by infection of adult flies through injection with

live *E. coli* or *S. aureus*, and monitoring fly survival as an effect of phagocytic ability (**figure 6A, p. 27**).

To test gene function *in vivo*, we used available viable null mutants (*simu*, *drpr*, *pgrp-lc*, *pgrp-sa*) or transgenic RNAi lines (VDRC Vienna, Dietzl et al. 2007) expressed in macrophages (*crq*Gal4) or glia (*repoGal4*). Whether a given gene was knocked down and tested in glia or macrophages depended on a gene's expression, which we inferred from our glial expression data combined with three S2 cell profiles from our lab, Michael Boutros, and Klaus Foerstermann, and our own RT-PCRs (**figure 7, p. 29; table 2, p. 29**). *UAS-dicer2* was co-expressed to enhance RNAi efficiency. If available, we used the latest generation of transgenic RNAi lines (KK), which carry the RNAi transgene in a specifically targeted genetic locus, and are therefore more likely to significantly decrease the overall rate of position effects leading to false positives and negatives. In addition, these transgenes carry introns, which enhance their RNAi efficiency and therefore reduce the number of false negatives. The KK lines are considered to present a significant improvement over the first generation of VDRC RNAi lines (GD lines), which, due to random insertion in the genome leading to variable transgene expression, were estimated to create phenotypes only in 60% of the cases (Dietzl et al. 2007).

3.1.1 *In vivo* apoptotic assay

In *Drosophila* development, apoptosis occurs in three major waves, in mid- to late embryogenesis, midpupa and then in the early adult (Thummel 2001). In particular the development of the nervous system generates many superfluous neurons, which have to be removed by phagocytes. While macrophages are responsible for apoptotic clearance during earlier stages of embryogenesis, the nervous system becomes ensheathed by the blood-brain-barrier at stage 16 and macrophages can no longer enter. Then the CNS-resident, astrocyte-like cell-body glia take over the task, as our lab and other studies have demonstrated (Sonnenfeld & Jacobs 1995; Kurant et al. 2008; M. R. Freeman et al. 2003). Along with the ectoderm, macrophages and glia are the three main phagocytic cell populations in the *Drosophila* embryo, and we can individually assess the clearance function of glia and macrophages in the embryo. Our initial goal was to find new glial factors, and our candidate selection for *in vivo* testing reflects that: 10 of the 18 chosen genes are upregulated in glia and were consequently tested *in vivo* in the glial assay in the embryo (**table 2, p. 29**). The remaining eight genes were bacterial receptors known to be expressed in macrophages or new genes, for which we presumed macrophage

expression based on the S2 expression data from Michael Boutros or RT-PCRs of whole embryos (**figure 7, p. 29; table 2, p. 29**). According to a gene's expression we knocked it down either in glia or in macrophages using specific drivers. In addition we used four available null mutants. To test apoptotic clearance in loss- of- function (lof) animals, we collected *Drosophila* embryos and aged them till stage 13 for the macrophage assay, when the macrophages can still freely move around and are not restricted yet in their range of motion by the developing CNS, or stage 16 for the glial assay, when the CNS is ensheathed and glia are responsible for apoptotic clearance (**figure 17, p. 56; figure 18, p. 65**). We were not interested in comparing macrophages to glia, but our goal was rather to find new players for both cell types, therefore we knocked down gene function either in glia or in macrophages. Embryos were fixed and stained with an antibody specific for activated Caspase-3 to detect apoptotic cells. To assess macrophage clearance of corpses in stage 13 embryos, we imaged whole embryos laterally using confocal microscopy and 50 slices were recorded with a total thickness of 57 µm. We analyzed glial phagocytosis by imaging the nervous system in stage 16 embryos and taking 35 confocal slices with a thickness of 17 µm. Confocal stacks were processed using Imaris 3D software creating isosurfaces around the apoptotic material, from which calculation of number and total volume of particles is possible. As controls, we tested the driver line *crqGal4; UAS-dicer* by itself as well as crossed to a seemingly non-functional RNAi line from the old (GD) library of VDRC lines, *UAS-Mdr65-RNAi*. As this line engages the RNA degradation machinery in a similar manner to the other RNAi lines, these flies have a more similar background to the test candidates and are therefore a more appropriate control. We imaged and analyzed 8-19 embryos for each genotype and assessed statistical significance using one-way ANOVA and Dunett's post test.

3.1.2 *In vivo* bacterial assays

Adult flies were aged for four or five days and injected with live *E. coli* or *S. aureus* (see 8.6 in Materia and Methods). We monitored fly survival as a read-out of phagocytic capacity, as previously reported (Bou Aoun et al. 2011; S. Meister et al. 2009; Shinzawa et al. 2009; Brandt et al. 2004; Cuttell et al. 2008; Clark & Bavoil 2002; Garver et al. 2006). *S. aureus* is highly pathogenic to flies and kills even wild-type flies with a t/2 of 4 days. However, removal of specific phagocytosis factors like the known Gram-positive recognition molecule PGRP-SA, which we used as a positive control in each experiment, increases the relative risk of death fourfold, with half of a cohort dead after 2.5 days (**figure 19A, p. 67**). In contrast, *E. coli* is not

pathogenic to *Drosophila* and does not affect viability of wild type flies, therefore any faster death of mutant flies reveals a gene's involvement in the process. A null mutation for the known *E. coli* receptor and Imd pathway effector *pgrp-lc* strongly reduces *Drosophila*'s immunity to *E. coli*. *pgrp-lc* flies have a survival t/2 of 2.5 days and were used as positive control in all experiments (**figure 20A, p. 68**). While we were able to confirm cell-based results *in vivo* using available null mutants, unfortunately we were not able to reproduce any effects from the cell-based screen using transgenic RNAi. The fact that not even transgenic RNAi of genes known to be required for immunity to *E. coli*, like *pgrp-lc*, led to any phenotype (**figure 20, p. 68**), suggests that transgenic RNAi does not lead to a phenocritical reduction of gene expression for this particular assay, and genes required for immunity to the weak pathogen *E. coli* can only be found in null conditions. For the genes we only had transgenic RNAi lines for, we therefore could not effectively assess their requirement in an *E. coli* infection *in vivo* and we can only make claims for the genes for which we had null mutants. To assess whether observed survival data were significantly different from each other, we employed the commonly used Cox regression analysis of survival returning P-values as well as the Relative Risk of Death (RRD) for members of a given test group/ genotype (Cox & Oakes 1984; Mair et al. 2005; Vigne et al. 2009).

3.2 *in vivo* versus *in vitro* results

We tested these 18 genes in two *in vivo* assays: apoptotic clearance in the embryo and adult infections with *S. aureus,* plus infections with *E. coli* for the four null mutants, totaling 40 tests (**figure 21, p. 84**). We were able to validate the cell-based results for the vast majority of cases (37/40 = 93%), showing that S2 cells serve as a useful tool to screen for phagocytosis candidates, and also that our mostly (RNAi derived) hypomorphic *in vivo* conditions were for the most part sufficient to create phenocritical states, where we could observe the effects of individual factors. How well did our return rate of *in vivo* versus cell-based compare to other screens? Although there is no phagocytosis screen that proceeded to test more than one or two candidates *in vivo*, we can compare *in vivo* confirmation rates with other studies using a combined cell-based an in *vivo* approach. two studies screened for regulators of the Notch pathway or Wingless secretion, respectively, first using tissue culture RNAi and then *in vivo* using transgenic RNAi and scoring eye and/ or wing phenotypes (Saj et al. 2010; Port et al. 2011). The Notch pathway study retested 501

genes *in vivo* and confirmed 59% of their cell-based results. The study investigating wingless secretion retested 115 genes *in vivo* and found 26% of genes from the cell-based screen to also have an effect *in vivo*. Our *in vivo* confirmation rate was higher than both of these screens (93%). These numbers show that while the transfer from cell-based assays to *in vivo* does not match up perfectly, combining cell-based and *in vivo* RNAi is a useful tool to find new factors in a given process.

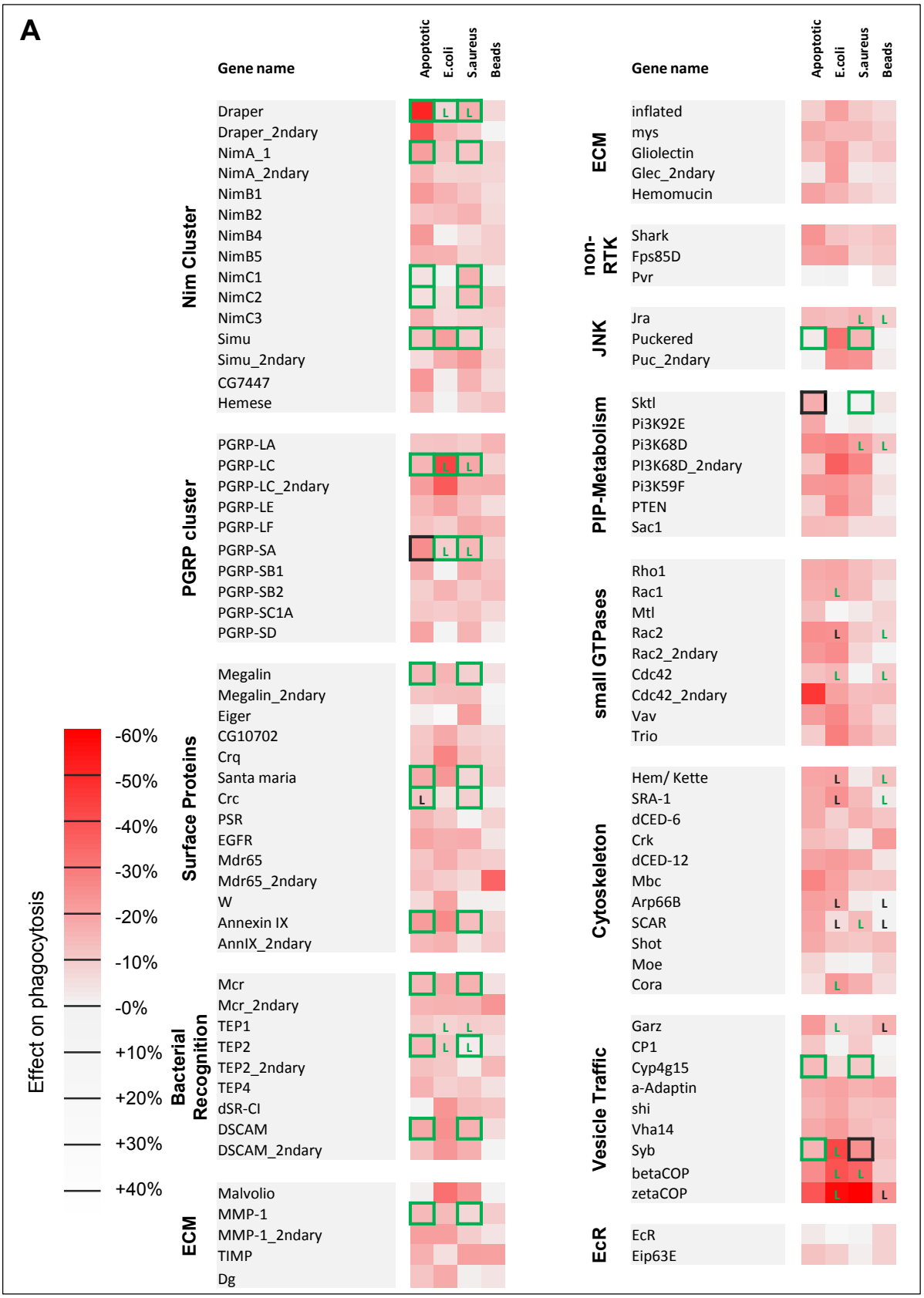
3.3 Consistency with literature

As a proof of principle, we included multiple genes that were known to play a role in phagocytosis of some sorts (**figure 15, p. 50**). These genes include constituents of the two *C. elegans* signaling cassettes, known cytoskeletal regulators and known receptors for apoptotic cells and bacteria. When we look at how our data compare to published results, we observe that consistency depends on the data source: results from other cell-based screens were mostly reproducible in terms of whether a given gene was considered required or not for a specific type of phagocytosis (24/38 tests = 63%, **figure 15A, p. 54**). When we look at the specific effect strengths of a given gene knockdown in different screens and compare the data to ours, RNAi of many genes, even though they are considered as having an effect in two independent studies, leads to significantly different effect strengths (**figure 2, p. 20**). For example *zCOP*: even though knockdown of this gene significantly inhibit phagocytosis of *E. coli* in three different studies, *zCOP* RNAi leads to 86% less *E. coli* engulfment in one study, 25% less engulfment in another and 52% less phagocytosis in my screen, pointing at the variability of the cell-based studies. However, when we compare our *in vivo* results with published data, which stem from in depth studies in null or hypomorphic conditions in model organisms including worm, fly and mouse, our data were reproduced without exception (23/23 tests = 100%, **figure 15B, p. 50**).

Following pages:

Figure 15: Results of cell-based phagocytosis screen and comparison with cell-based and in vivo results from the literature.

Displayed are effects of RNAi knockdowns of candidate genes for the different food types. Shown are means of 5-14 replicates. A green box indicates consistency with the in vivo *result, black boxes indicates discrepancy. Green L's indicate consistency with cell-based results (**A**) or* in vivo *results (**B**) from the literature, black L's discrepancy.*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Gene name
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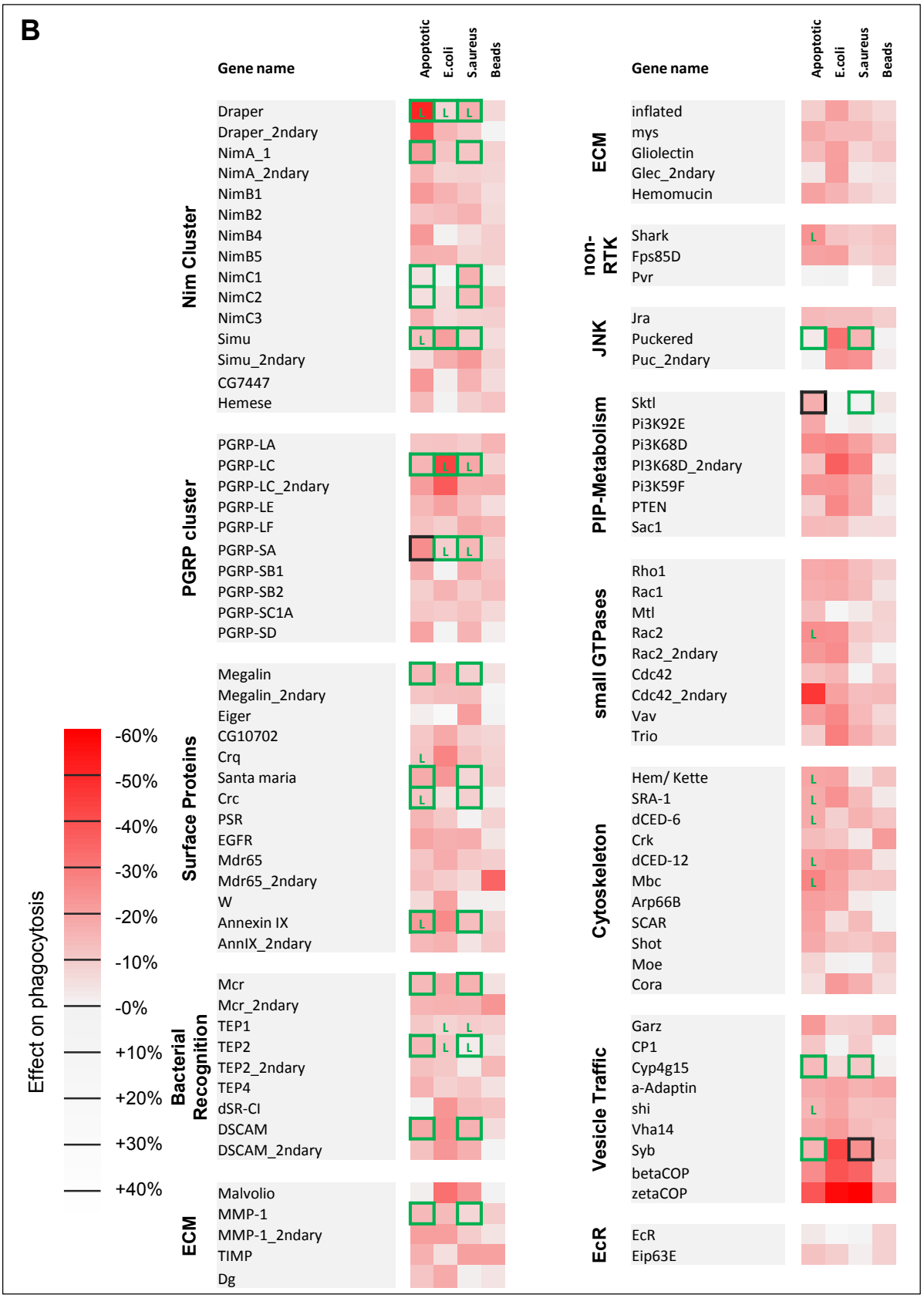
E.coli
S.aureus
Beads
Nim Cluster
Draper
Draper_2ndary
NimA_1
NimA_2ndary
NimB1
NimB2
NimB4
NimB5
NimC1
NimC2
NimC3
Simu
Simu_2ndary
CG7447
Hemese
PGRP cluster
PGRP-LA
PGRP-LC
PGRP-LC_2ndary
PGRP-LE
PGRP-LF
PGRP-SA
PGRP-SB1
PGRP-SB2
PGRP-SC1A
PGRP-SD
Surface Proteins
Megalin
Megalin_2ndary
Eiger
CG10702
Crq
Santa maria
Crc
PSR
EGFR
Mdr65
Mdr65_2ndary
W
Annexin IX
AnnIX_2ndary
Bacterial Recognition
Mcr
Mcr_2ndary
TEP1
TEP2
TEP2_2ndary
TEP4
dSR-CI
DSCAM
DSCAM_2ndary
ECM
Malvolio
MMP-1
MMP-1_2ndary
TIMP
Dg
Effect on phagocytosis
-60%
-50%
-40%
-30%
-20%
-10%
-0%
+10%
+20%
+30%
+40%
ECM
inflated
mys
Gliolectin
Glec_2ndary
Hemomucin
non-RTK
Shark
Fps85D
Pvr
JNK
Jra
Puckered
Puc_2ndary
PIP-Metabolism
Sktl
Pi3K92E
Pi3K68D
PI3K68D_2ndary
Pi3K59F
PTEN
Sac1
small GTPases
Rho1
Rac1
Mtl
Rac2
Rac2_2ndary
Cdc42
Cdc42_2ndary
Vav
Trio
Cytoskeleton
Hem/ Kette
SRA-1
dCED-6
Crk
dCED-12
Mbc
Arp66B
SCAR
Shot
Moe
Cora
Vesicle Traffic
Garz
CP1
Cyp4g15
a-Adaptin
shi
Vha14
Syb
betaCOP
zetaCOP
EcR
EcR
Eip63E


B
Gene name
Apoptotic
E.coli
S.aureus
Beads
Nim Cluster
Draper
Draper_2ndary
NimA_1
NimA_2ndary
NimB1
NimB2
NimB4
NimB5
NimC1
NimC2
NimC3
Simu
Simu_2ndary
CG7447
Hemese
PGRP cluster
PGRP-LA
PGRP-LC
PGRP-LC_2ndary
PGRP-LE
PGRP-LF
PGRP-SA
PGRP-SB1
PGRP-SB2
PGRP-SC1A
PGRP-SD
Surface Proteins
Megalin
Megalin_2ndary
Eiger
CG10702
Crq
Santa maria
Crc
PSR
EGFR
Mdr65
Mdr65_2ndary
W
Annexin IX
AnnIX_2ndary
Bacterial Recognition
Mcr
Mcr_2ndary
TEP1
TEP2
TEP2_2ndary
TEP4
dSR-CI
DSCAM
DSCAM_2ndary
ECM
Malvolio
MMP-1
MMP-1_2ndary
TIMP
Dg
Effect on phagocytosis
-60%
-50%
-40%
-30%
-20%
-10%
-0%
+10%
+20%
+30%
+40%
Gene name
Apoptotic
E.coli
S.aureus
Beads
ECM
inflated
mys
Gliolectin
Glec_2ndary
Hemomucin
non-RTK
Shark
Fps85D
Pvr
JNK
Jra
Puckered
Puc_2ndary
PIP-Metabolism
Sktl
Pi3K92E
Pi3K68D
PI3K68D_2ndary
Pi3K59F
PTEN
Sac1
small GTPases
Rho1
Rac1
Mtl
Rac2
Rac2_2ndary
Cdc42
Cdc42_2ndary
Vav
Trio
Cytoskeleton
Hem/ Kette
SRA-1
dCED-6
Crk
dCED-12
Mbc
Arp66B
SCAR
Shot
Moe
Cora
Vesicle Traffic
Garz
CP1
Cyp4g15
a-Adaptin
shi
Vha14
Syb
betaCOP
zetaCOP
EcR
EcR
Eip63E

4 Screen outcomes

4.1 Cell-based screen

We tested 86 candidate genes belonging to different cellular compartments and processes for their involvement in phagocytosis of apoptotic cells, bacteria and beads. We recovered many known genes including constituents of the two *C. elegans* signaling cassettes, and other cytoskeletal regulators that were known to be important for phagocytosis. We also found genes known from *Drosophila* phagocytosis including *simu*, *drpr* and *nimC1* (**figure 10, p. 36; figure 14, p. 45**), all of which had previously been shown to play a role in the process. Interestingly, we also found novel functions for some of these genes in cases other than the previously reported type of phagocytosis. For example we were surprised to find that *simu* is not only necessary for phagocytosis of apoptotic cells, but also for phagocytosis of *S. aureus*; and *pgrp-lc* not only for *E. coli* phagocytosis, but also for apoptotic cells. This cross-specificity of factors, especially surface receptors or secreted molecules known to recognize either bacteria or apoptotic cells, was an intriguing and unexpected finding and pertained to a large portion of the tested genes (**figure 16, p. 54**), prompting us to seek *in vivo* validation. Apart from novel functions for previously known genes, we also found new phagocytic functions for genes that were known but had never been implicated in this process before, or entirely novel genes without any known function. Two examples for such novel players are *nimA* and *cyp4g15* **(figure 10, p. 36).** *nimA* belongs to the NIM family of genes, which also contains *simu* and *drpr*, but *nimA* itself has never been implicated in phagocytosis. *cyp4g15* is a gene without any known function, but based on its homology it is annotated as a cytochrome P450 and it is highly expressed in embryonic glia. Here, we report that both genes are specifically necessary for apoptotic cell phagocytosis, but not for phagocytosis of bacteria or beads, and we validate these gene functions *in vivo*.

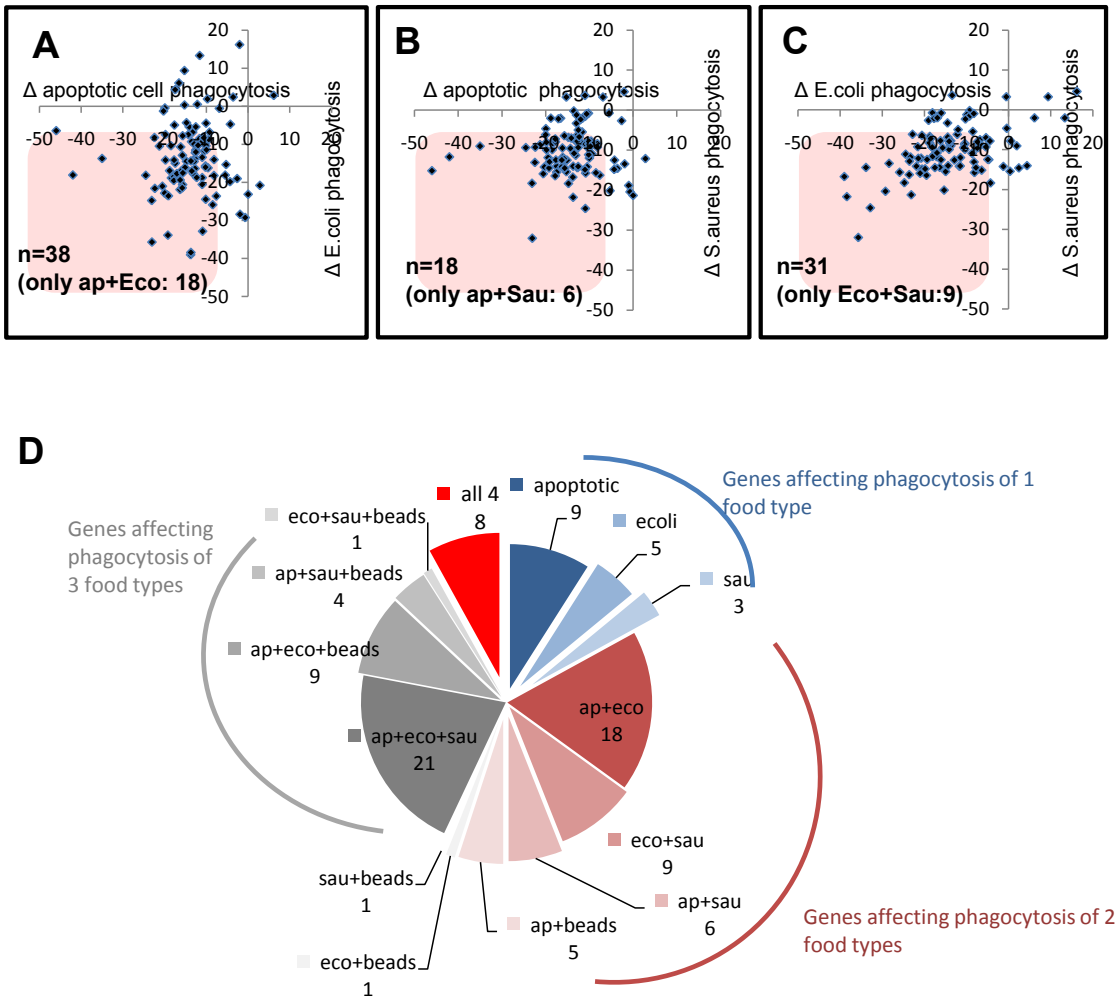


Figure 16: Cross-specificity of phagocytosis genes – cell-based screen.

A-C *Effects of specific gene knock downs on phagocytosis in the cell-based screen are plotted. The axes represent the effects on phagocytosis of a specific food type. The lower left quadrant indicates significant reductions greater than 10% on both plotted food types. The number of genes in the quadrant is indicated as well as the number of genes exclusively affecting a given pair of food types.* ***A*** *x-axis: effect on phagocytosis of apoptotic cells, y-axis: effect on phagocytosis of* E. coli. *38 genes significantly reduce both types of phagocytosis by at least 10 %, and out of these, 18 genes affect only apoptotic cell and* E. coli*, but not the other types of phagocytosis.* ***B*** *x-axis: effect on phagocytosis of apoptotic cells, y-axis: effect on phagocytosis of* S. aureus. ***C*** *x-axis: effect on phagocytosis of* E. coli*, y-axis: effect on phagocytosis of* S. aureus. ***D*** *Pie chart displaying numbers of genes affecting single food types or combinations of foods. Genes having a significant effect of more than 10% are shown. The blues show factors specific to only one type of food. The reds show genes affecting 2 types of food. The greys show numbers of genes affecting different combinations of 3 food types. Bright red, 8 genes are required for all 4 tested types of phagocytosis.*

4.2 *In vivo* testing

The process of phagocytosis had not previously been systematically compared for different food types, including apoptotic cells. The 18 genes we selected for *in vivo* testing, as in the cell-based screen, are associated with different cellular compartments from the surface to more downstream events in the phagocytic process (**figure 21, p. 84**). Using this approach, we were now also hoping to functionally dissect phagocytosis of different types of food *in vivo* using two suitable paradigms: developmental clearance of apoptotic neurons and adult clearance of infection in the immune system. Another aspect was finding new factors for glia and macrophages, both of which are specialized to eat dying cells in the developing embryo. Many of the genes chosen for the primary cell-based and then for the secondary *in vivo* screen are upregulated in glia and were therefore also knocked down in glia *in vivo* using the *repoGal4* driver and checked for their involvement in the process. Non-glial genes were knocked down in macrophages using the *crqGal4* driver. For four of the 18 genes we were able to use available null mutants. Genes were selected along a cross-section through the cell, from the cell surface to 'core' machinery and signaling events. Specifically, the genes chosen include the members of the NIM gene cluster, the PGRP gene family, other surface molecules, bacterial recognition genes, a matrix metalloproteinase, and genes from vesicle trafficking (**figure 21, p. 84**). Thus, the candidates reflect different aspects of the phagocytic process, from recognition through different types of mechanisms and molecules, to signaling and phagosome maturation.

5 Novel players in *Drosophila* glial phagocytosis

We tested a number of interesting genes, selected on the basis that they were highly expressed or even upregulated in glia, showed an effect in the cell-based screen and/or were orthologs to known phagocytosis factors from vertebrates, or shared sequence similarity to *Drosophila* genes known to be involved in phagocytosis.

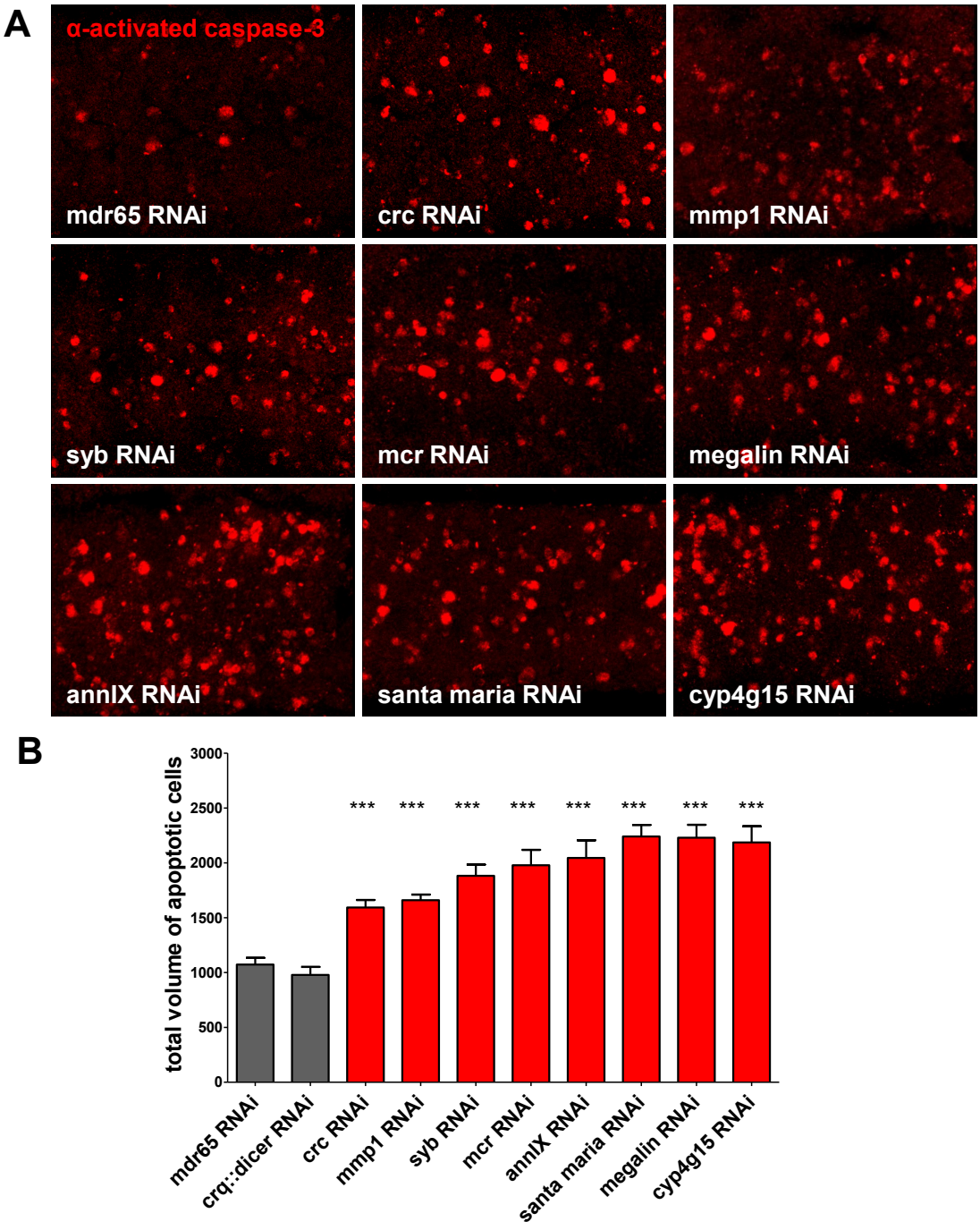


Figure 17: Glial clearance of apoptotic cells - novel players.

*Embryos of flies with RNAi transgenes expressed in glia (*repo-G4*) were fixed, stained for activated Caspase 3 (α-CM1) and imaged using confocal microscopy.* ***A*** *Images represent segments 3-6 of the nervous system in stage 16 embryos. Depicted are confocal stacks of 17.5 µm.* ***B*** *Total volume of apoptotic particles was measured using Imaris 3D isosurfacing. Bar charts represent total volume of apoptotic material. 1-way ANOVA to test for significance. *** P < 0.0001*

5.1 Bridging molecules and receptors

The mammalian Annexin family comprises 12 calcium or calcium and phospholipid binding proteins that perform a range of functions inside and outside cells, including intracellular calcium signaling and membrane scaffolding and extracellular fibrinolysis and immune signaling modulation. Certain Annexins assume a special role in apoptotic clearance, because they bind newly exposed PS on the apoptotic cell surface. It is through this opsonization that the corpse is labeled for phagocytosis (Gerke et al. 2005). It has been shown that both Annexin (ANN) I and V bind PS, and *C. elegans* deficient for *annI* show defects in phagocytosis of apoptotic cells, confirming the important role of Annexins in apoptotic clearance (Arur et al. 2003). The ANN-PS complex is arguably recognized by a PS receptor on macrophages, which subsequently engulf the apoptotic cell (Mitchell et al. 2006; Williamson & Schlegel 2004). Annexins are conserved throughout evolution and are present in all clades of the animal and plant kingdoms. *Drosophila* possesses three Annexins, IX, X and XI. Even though Annexins are presumably ubiquitous due to their role in basic processes like calcium signaling and membrane organizing, two of the *Drosophila* Annexins – IX and X – are upregulated in glia, according to our expression profiling. When we tested *annIX* in our cell-based assay we observed a strong reduction in phagocytosis of apoptotic cells, suggesting that this gene also plays a role in the process in *Drosophila* (**figure 14, p. 45**). Transgenic RNAi of *annIX* in glia confirmed this observation: embryos with a knock down of this factor in glia showed an accumulation of corpses in the embryonic nervous system (**figure 17, p. 56**). This shows that *annIX* is required for phagocytosis of apoptotic cells in *Drosophila* as well, underlining the evolutionary conservation of this bridging molecule and its important role in labeling unwanted cells for engulfment.

Another known player from vertebrate cells is *crc*. This classical ER protein does not only act as an ER-resident chaperone, but has also been found to be involved in various other processes including adhesion, migration, immunomodulation and phagocytosis (Gold et al. 2010). In particular it has been shown that CRC can act *in*

trans as an 'eat-me' signal exposed on apoptotic cells as well as *in cis* as a receptor for collectins binding apoptotic cells on the phagocyte (Gardai et al. 2005; Vandivier et al. 2002). *crc* knockdown had a mild effect on our cell-based apoptotic clearance assay (**figure 14, p. 45**), but transgenic RNAi in glia *in vivo* magnified this finding: *crc* is highly upregulated in glia, and when we knocked down gene function using transgenic RNAi we observed that *crc*-deficient glia have a reduced capacity for engulfment, leading to an accumulation of apoptotic material in the embryonic nervous system (**figure 17, p. 56**). This increase is also interesting, because while highly significant, the magnitude was only 1.5 fold, which is somewhat less than for other tested factors. This suggests that either knockdown efficiency was low or *crc* function can be at least partly compensated by another gene. Finding *annIX* and *crc* to be involved in the process was expected but nevertheless exciting, because it shows that corpse engulfment is, despite its increasing complexity, an evolutionarily conserved process and governed by the same factors in invertebrates and vertebrates.

Finally, we tested two genes based on their sequence similarity with known phagocytosis factors in *Drosophila*: *megalin* and *scavenger receptor acting in neural tissue and majority of rhodopsin is absent* (*santa maria)*. One of the 'eat-me' signals newly exposed on apoptotic cells is oxidized low density lipoprotein (LDL), which is in turn recognized by specific receptors on phagocytes (Oka et al. 1998; Boullier et al. 2001). One of the identified receptors in this process is *cd36*; removal of this factor has been shown to play a role in apoptotic cell clearance in vertebrates, where transfection of *cd36* into non-phagocytic cells rendered them able to phagocytose apoptotic cells (Greenberg et al. 2006). A *Drosophila* homolog of *cd36* is *crq*, which is expressed in embryonic macrophages and is required for phagocytosis of apoptotic cells (Franc, Heitzler, et al. 1999). We found a closely related gene, *santa maria* (82% sequence identiy), to be additionally highly expressed in glia and tested its role in the process. *santa maria* was strongly required for phagocytosis of apoptotic cells *in vitro* and *in vivo*, showing that *santa maria* has the same function in glia as in macrophages (**figure 14, p. 45, figure 17, p. 56**). *crq* and *santa maria* therefore seem to have evolved from a common ancestor gene, but both receptors preserved their function in corpse clearance, with the difference that *santa maria* (but not *crq)* is additionally expressed in glia. It remains to be elucidated why the same receptor is not reused for both cell types, and why glia do not express *crq,* but *santa maria* instead.

Another intriguing *cd36* homolog specifically upregulated in embryonic glia is *megalin*. In addition to its similarity to *cd36, megalin* contains EGF-like domains, which are also present in the known phagocytosis receptor *drpr*, making *megalin* an overall interesting candidate. We knocked down gene function in embryonic glia and found that phagocytosis of apoptotic cells was indeed significantly reduced in the nervous system, resulting in many more lingering apoptotic particles (**figure 17, p. 56**). We thus showed for the first time that the *Drosophila megalin* acts as a receptor for apoptotic cells on glia in the developing nervous system, underlining the general role of *cd36*-like proteins in apoptotic cell recognition.

5.2 Extracellular matrix

The ECM is a crucial element in all animal body plans. It lines all animal organs and tissues and provides order and structural integrity to the living organism. It consists of a complex meshwork of hydrophilic proteoglycans and glycoproteins promoting cell adhesion and cell signaling, anchoring cells and giving them orientation as well as survival or death signals. During normal developmental processes the ECM must be altered in order for metamorphosis, tissue remodeling or migration through barriers to occur, and ECM molecules are sensed by adjacent cells through a number of receptors including integrins and Dystroglycan. Degradation of the ECM is associated with many diseases including cancer growth and metastasis (Page-McCaw et al. 2007). As many ECM receptors and associated factors are highly upregulated in glia, we included a subset of these genes in our phagocytosis screen - another process integral to tissue remodeling and homeostasis. While the integrin *myospheroid* and the lectin receptor *gliolectin* showed involvement in the process, we decided to focus on the intriguing finding that the *matrix metalloproteinase 1* (*mmp1*) seemed to be required for phagocytosis of apoptotic cells (**figure 10, p. 36**). The MMP family of proteolytic enzymes can cleave almost every component of the ECM, and its members are implicated in many ECM remodeling processes during healthy and abnormal development and homeostasis. While the mammalian genomes contain over 20 MMPs that have mostly redundant functions, *Drosophila* has only two, *mmp1* and *mmp2*, making the functional dissection of these genes feasible. It has been shown that null mutants for either of the fly MMPs leads to aberrant axon defasciculation in the embryo, perturbing normal nervous system development. (C. Miller et al. 2008; Yasunaga et al. 2010). *mmp1* has been shown to be a secreted molecule, which is expressed in S2 cells as well as in the embryonic

CNS. In our glial expression profiling we found *mmp1* to be highly expressed in embryonic glia, and we decided to test its involvement in phagocytosis of apoptotic cells. Our cell-based screen showed a requirement for *mmp1* in phagocytosis of apoptotic cells, so we decided to check its *in vivo* function in glia. RNAi of the secreted molecule in glia indeed confirmed the cell-based finding: apoptotic cells accumulate sustainedly in the embryonic CNS due to a defect in their phagocytosis by glial cells (**figure 17, p. 56**). Vertebrate cell-culture studies had previously shown that MMPs can play a role in phagocytosis: in addition to the extracellular degradation of the ECM during remodeling processes, phagocytic degradation can occur as well (H. Lee et al. 2007). However, it is unclear whether the function of secreted *mmp1* is indeed akin to a phagocytic receptor, which would necessitate another recognition molecule on the phagocyte's – the glial – surface, or whether the protease is instead involved in shearing away the ECM in the CNS providing the necessary freedom of movement for the glia to engulf their apoptotic targets. In any event, this is the first time a glial-expressed *mmp* has been implicated in developmental clearance of apoptotic cells, pointing at the importance of phagocyte-matrix interactions during phagocytosis.

5.3 Signaling and phagosome maturation

During phagocytosis, it is not enough to just recognize what is to be eaten. The cell subsequently needs to perform complex actions to enlarge its surface, stretch its 'arms' and enwrap its target, which requires coordinated signaling and major cytoskeletal reorganization. The final stage of phagocytosis is phagosomal maturation and degradation of the contained particle into its basic molecular units. Proton pumps integrated in the phagosomal membrane acidify the phagosome lumen and thereby create the right digestive milieu to dissolve all phagosomal content. To this end the phagosome is enriched with proteo-, glyco- and lipolytic enzymes, which acquire their hydrolytic capacities at low pH. How is this change in phagosomal composition achieved? Phagosomes repeatedly interact with vesicles from the endocytic pathway through consecutive fusion and fission events. In this fashion the phagosome changes and matures; early endo-and phagosomal factors are segregated away into parting vesicles, while late endo- and phagosomal factors are delivered by arriving vesicles. These membrane fusion events are executed by special membrane proteins on the vesicle and target membranes, SNAREs, forming alpha-helical bridges *in trans* that increase membrane curvature and induce lipid

bilayer breaks, which eventually result in vesicle fusion or fission (Zhou & Yu 2008; Underhill 2005). One of the core SNARE machinery members is Synaptobrevin (SYB) that has been initially described and studied in the context of fast neurotransmitter release from presynaptic vesicles at the chemical synapse. *syb* however is required not only for exo-, but also for recycling endocytosis of neurotransmitters at the axonal endplate and is generally part of the core membrane fusion complex consisting of four SNARE proteins (Jahn & Südhof 1999; Deak et al. 2004). While *syb*'s role in neurotransmitter exocytosis had been extensively studied, we were surprised to find it highly upregulated in embryonic glia and went on to test its possible involvement in phagocytosis, an entirely different process that nevertheless requires multiple fusion and fission events as well. The cell-based results indeed showed a broad requirement for *syb* in phagocytosis of all tested targets, pointing to an important role of this gene in phagocytosis (**figure 14, p. 45**). *In vivo* testing of a transgenic RNAi line expressed in glia confirmed this result: knock down of *syb* in glia leads to a significant accumulation of apoptotic cells in the embryonic CNS (**figure 17, p. 56**). This result demonstrates for the first time that *syb* is not only required in neurons for synaptic transmission, but is also involved it in glial function. Here, it is a necessary factor in the engulfment process, probably through delivery of vesicles to the plasma membrane thereby increasing the phagocytic cup surface.

The cytochrome P450 superfamily comprises a large number of enzymes that catalyze monooxidation of substrates with the help of the hem cofactor. They are crucial for different metabolic processes including steroid hormone biosynthesis and drug metabolism, where they account for ca. 75% of all metabolic reactions (Adams et al. 2000; Maïbèche-Coisne et al. 2000). We found *cyp4g15* to show strong expression in embryonic glia, suggesting a function in these cells. To test this hypothesis we knocked down gene function in glia using transgenic RNAi and saw that this indeed led to a strong increase of apoptotic material in the nervous system (**figure 17, p. 56**). This shows that *cyp4g15* is required for corpse phagocytosis, an entirely novel function for *cyp4g15* enzymes and cytochromes in general. The exact function of Cyp4g15 in phagocytosis of apoptotic cells remains to be elucidated.

In summary, we successfully tested a set of factors for their involvement in developmental corpse clearance by glia. We found homologs of vertebrate factors, as well as identified new *cd36*-family members as players in this highly redundant

process, shedding light on how glia manage to quickly recognize and remove apoptotic material during brain development.

6 Cross-specificity of phagocytic factors

Many different factors have been implied in various – mostly cell-based – model systems in phagocytosis of apoptotic cells, bacteria, beads and other types of material. The proteins involved have very different structures and features, and many appear to be promiscuous in their ligand choice, binding not only bacteria, but also apoptotic cells. However, these findings were rarely made under the same conditions, and a systematic evaluation has been lacking. When we found many of the genes tested in S2 cells to be cross-specific for different food types, we tested the most interesting candidates *in vivo*.

6.1 Opsonins

The first step in phagocytosis is target recognition: a cell needs to sense its food to start engulfing it. The longest-range sensors are not receptors, but secreted molecules that float in the blood/ haemolymph and bind to phagocytic targets. These opsonins have been described in much detail for bacterial recognition, and a major class of opsonins are the thioester-containing proteins of the vertebrate complement cascade. These factors recognize and bind molecular patterns on the bacterial surface, and are in turn recognized by integrin-based complement receptors on the phagocyte (Philippe 2004; Danilova 2006; Dzik 2010). Interestingly, *Drosophila* also has a class of thioester-containing proteins, the TEPs. It consists of six genes, *tep1-6*, in which *tep5* seems to not be expressed and *tep6* is commonly known as macroglobulin complement related (*mcr,* Blandin & Levashina 2004). *tep2* and *mcr* had been implicated in *E. coli* phagocytosis by S2 cells, but *in vivo* lof of individual TEPs failed to produce any phenotypes, raising the possibility that these proteins can compensate for each other (Bou Aoun et al. 2011; Stroschein-Stevenson et al. 2006). We tested *tep1,2,4* and *mcr* in our screen, and confirmed the requirement for *tep2* and *mcr* for *E. coli* phagocytosis. *tep2* does not play a role in *S. aureus* phagocytosis, as had been shown before, but we surprisingly observed that *mcr* is required for uptake of this bacteria (**figures 14, 15, p. 45, 50**). This is in line with our own *in vivo* validation, which had never been performed before: transgenic RNAi in macrophages leads to a significant acceleration of death in adult flies infected with live *S. aureus*, revealing a novel role for this factor in bacterial phagocytosis (**figure 19, p. 67**). Moreover, we were very surprised to find that both *tep2* and *mcr* also seem to be required for apoptotic cell phagocytosis, and embryonic analysis of apoptotic clearance confirms these findings: lof of *mcr* as well as *tep2* in

macrophages leads to a significant increase of apoptotic material in the embryo (**figure 18, p. 65**). This shows that these complement-like factors are not specifically recognizing bacteria and yeast, as has been postulated, but are also promoting engulfment of apoptotic cells. While complement binding to apoptotic cells has been shown in vertebrate cells, we demonstrate here for the first time *in vivo* that complement opsonization is a mechanism for engulfment of apoptotic cells.

6.2 The NIM family

The NIM gene family of secreted or transmembrane proteins was recently identified as a novel class of engulfment receptors (Kurucz et al. 2007). It contains the apoptotic cell receptors *simu* and *drpr*, but also the *S. aureus* receptor *nimC1* and *eater*, which is required for phagocytosis of both *S. aureus* and *E. coli* phagocytosis, suggesting that the *nims* evolved and diversified from their common worm ancestor *ced-1* into a family of functionally distinct phagocytosis receptors (**figure 3, p. 22**). However, only three out of the thirteen members have been tested and implicated in phagocytosis so far. Because of their different specificities – *simu* and *drpr* for apoptotic cells, and *nimC1* for Gram-positive bacteria – we were interested to systematically test the NIM family in all our assays: apoptotic cells, Gram-negative, Gram-positive and bead eating. We recovered the known functions of NIM genes, but also uncovered novel ones (**figures 14, p. 45; figure 15, p. 50**). One gene that is necessary for apoptotic cell clearance in S2 cells, *nimA* (**figure 10, p. 36**), was of particular interest to us, as it is most closely related to *drpr* and therefore seemed a good candidate for *in vivo* testing. *nimA* contains, similar to *drpr*, an N-terminal EMI domain, followed by one NIM repeat and two EGF-like repeats; it also, like *drpr*, contains a large intracellular portion (**figure 3, p. 22**).

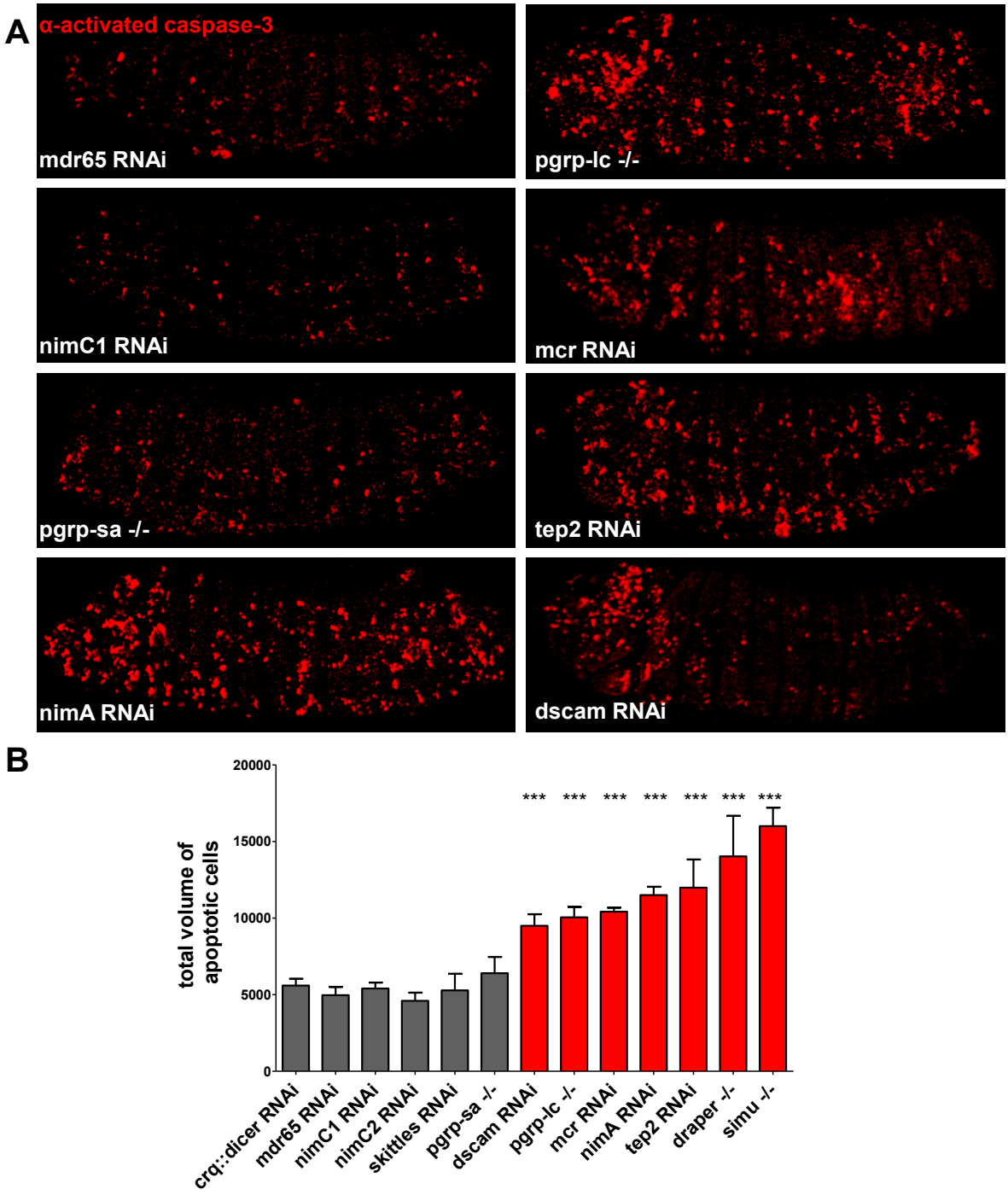


Figure 18: Macrophage clearance of apoptotic cells - novel players.

*Embryos of flies with RNAi transgenes expressed in macrophages (*crq-G4*) or null mutants were aged, fixed, stained for activated Caspase 3 (α-CM1) and imaged using confocal microscopy.* ***A*** *Whole stage 13 embryos are imaged in lateral view. Shown are confocal stacks of 57 µm.* ***B*** *Total volume of apoptotic particles was measured using Imaris 3D isosurfacing. Bar charts represent total volume of apoptotic material. 1-way ANOVA to test for significance. *** P < 0.0001*

nimA is expressed in the embryo (**figure 7**) and we found that transgenic RNAi of *nimA* in embryonic macrophages led to a strong increase of apoptotic material in the *Drosophila* embryo (**figure 18, p. 65**). This confirms our cell-based findings and identifies *nimA* as yet another *ced-1* homolog involved in apoptotic clearance. As suggested by the cell-based results, we did not find *nimA* to play a role in bacterial clearance (**figure 19, p. 67; figure 20, p. 68**), showing that it is a specific recognition molecule of apoptotic cells.

A gene which was, however, required for bacterial eating was *nimC2*, a close paralog to *nimC1*, which is a known receptor for Gram-positive *S. aureus*. We found *nimC2* to have a phagocytosis profile very similar to *nimC1*, which is exclusively required for *S. aureus* clearance (**figures 10, 14, 15A**), and therefore went on to test *nimC2 in vivo*. Indeed we found that *nimC2* knockdown in macrophages increases the flies' susceptibility to *S. aureus* infection (**figure 19, p. 67**), but has no effect on apoptotic cell phagocytosis (**figure 18B, p. 65**). This identifies *nimC2* as a novel specific receptor for Gram-positive *S. aureus*.

In summary, we newly identified two specific receptors for very different food types through our screen, *nimA* for apoptotic cells and *nimC2* for *S. aureus*, manifesting the important function the NIM family plays in different aspects of phagocytosis, but also pointing at the immense redundancy of phagocytosis factors and the general importance of the process. While both *nimA* and *nimC2* seem to be specific for their respective food types, we made a rather surprising observation regarding our players *simu* and *drpr*: both genes seemed not only to be required for apoptotic cell eating, as we knew already, but also seemed to affect phagocytosis of *S. aureus* and *E. coli* (**figure 10, p. 36; figure 14, p. 45; figure 15, p. 50**). To follow up these puzzling findings, we tested *simu* and *drpr* null mutants *in vivo* in our bacterial infection assay. We could indeed validate the cell-based results *in vivo*: particularly immunity towards *S. aureus* is strongly compromised in *simu* or *drpr* nulls, which die as fast as the positive control (flies deficient in the Gram-positive peptidoglycan receptor *pgrp-sa*), pointing at SIMU and DRPR's ability to recognize Gram-positive bacteria (**figure 19, p. 67**).

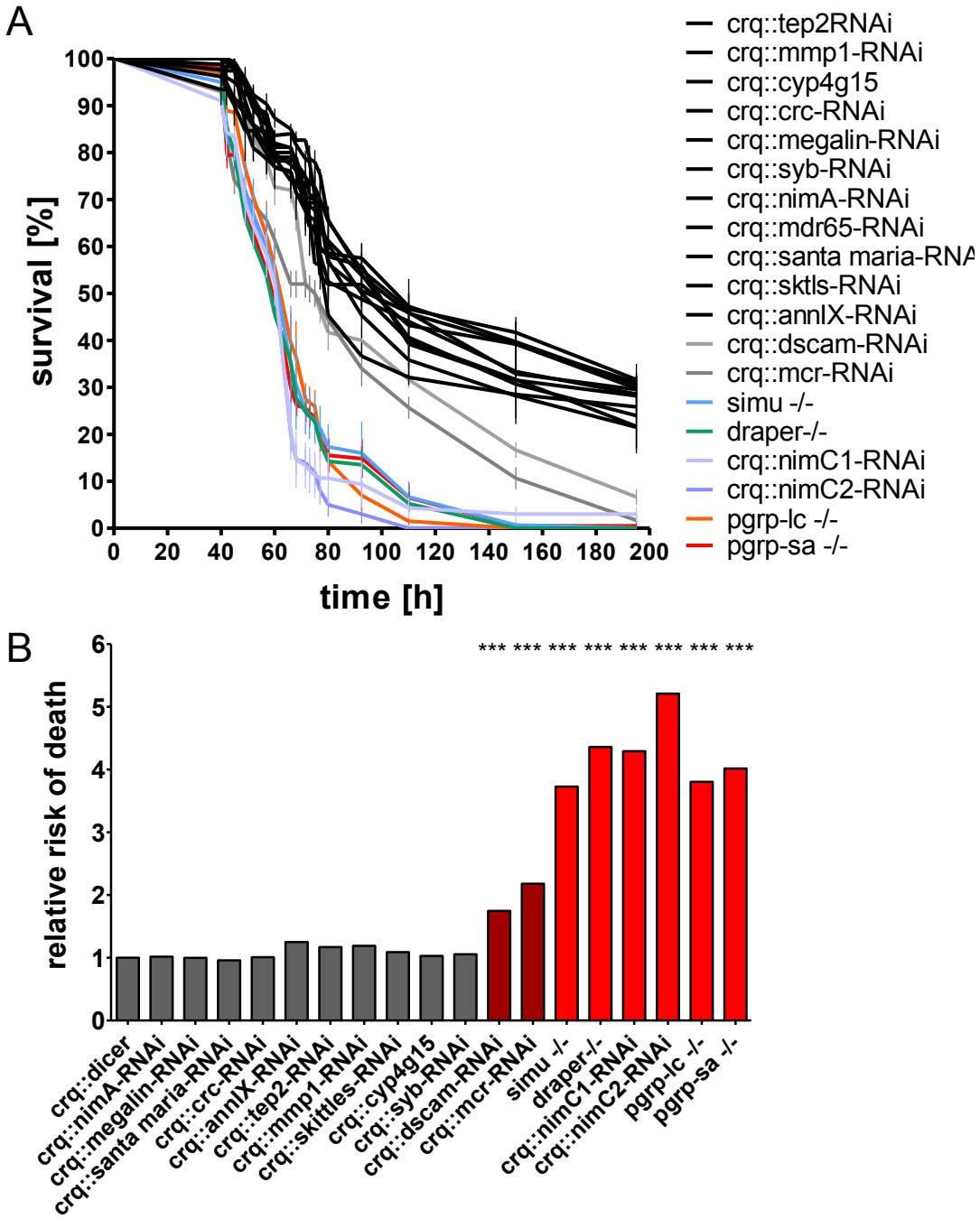


Figure 19: Novel players in bacterial clearance - S. aureus.

*Adult flies with RNAi transgenes expressed in macrophages (*crq-G4*) or null mutants were injected with diluted overnight cultures of* S. aureus *and survival was monitored as a read-out of phagocytic capacity.* crq::mdr-65RNAi *flies were used as control. Shown is the mean of 3-5 independent experiments with the the SEM.* ***A*** *Loss-of-function of specific genes increases lethality of* S. aureus *infected flies. A significant decrease of survival is indicated in red.* ***B*** *Relative risk of death for flies in indicated genotype groups. Significance and Relative Risk of Death were assessed using the Cox regression survival analysis. *** P < 0.0001*

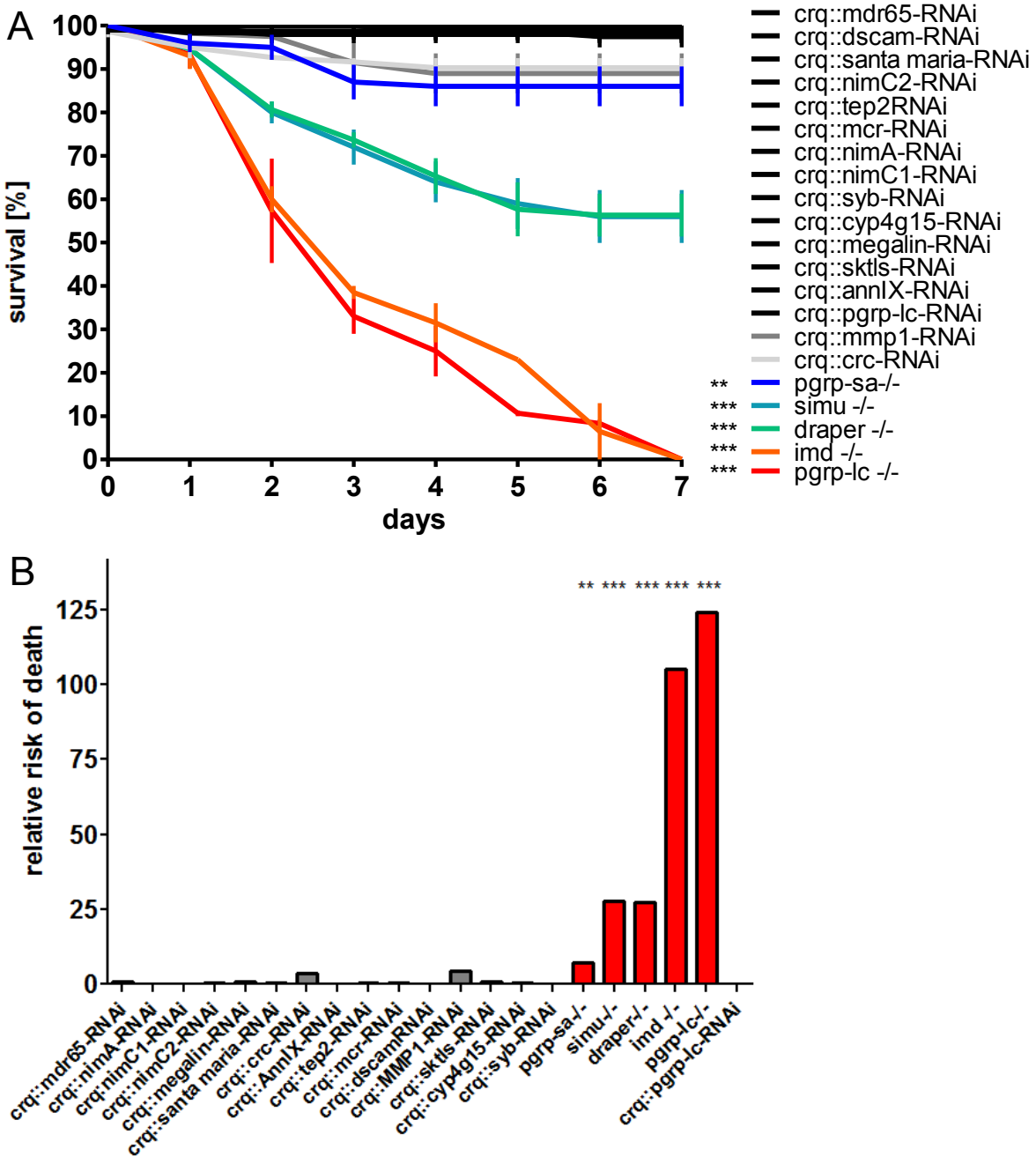


Figure 20: Novel players in bacterial clearance- E.coli.

*Adult flies with RNAi transgenes expressed in macrophages (*crq-G4*) or null mutants were injected with overnight cultures of* E. coli *and survival was monitored as a read-out of phagocytic capacity.* crq::mdr-65RNAi *flies were used as control. Shown is the mean of 3-5 independent experiments with the SEM.* ***A*** E. coli *is non-pathogenic to healthy flies, but kills flies with loss-of-function of specific macrophages genes. A significant decrease of survival is indicated in red.* ***B*** *Relative risk of death for flies in indicated genotype groups. Significance and Relative Risk of Death were assessed using the Cox regression survival analysis. ** P<0.001, *** P < 0.0001.*

Both genes also affected *Drosophila*'s immunity to *E. coli*. While control flies are completely immune to *E. coli* infection and remain unaffected by it, *drpr* and *simu* nulls become susceptible to the infection and start dying after one day, with the maximal death in a cohort reaching 45% after one week; at this point control flies are all still alive and the positive control, flies deficient in the Gram-negative peptidoglycan recognition protein *pgrp-lc,* are all dead **(figure 20, p. 68**). Removal of *simu* or *drpr* has a relatively weak effect on immunity to *E. coli* infection compared to the positive control, *pgrp-lc*, and most *simu* or *drpr* flies remain unaffected by the infection. However, the contribution by both receptors to immunity against *E. coli* is significant. These findings are very intriguing, as both genes had only been discussed and studied in apoptotic cell clearance paradigms. Their additional involvement in bacterial clearance uncovers a fundamental cross-specificity of phagocytosis factors, which through as yet unknown interactions are able to recognize ligands on targets as divergent as eukaryotic apoptotic cells and prokaryotic bacteria.

6.3 The PGRPs

The PGRPs are a famous class of bacterial recognition proteins (Royet & Dziarski 2007; Dziarski & Gupta 2006). The PGRP genes form clusters in the genome and are conserved throughout evolution from molluscs to mammals. In *Drosophila* there are 13 PGRPs, with some of them having multiple splice isoforms. There are short (S) and long (L) forms, and secreted and transmembrane PGRPs, but all share a peptidoglycan recognition domain, and differences in this domain determine ligand specificity: the secreted PGRP-SA, -SD and GNBP1 all recognize the Lysin-type proteoglycan of Gram-positive bacteria, while the membrane-bound PGRP-LC recognizes DAP-type proteoglycan of Gram-negative bacteria. Interestingly, these receptors not only recognize different types of bacteria, but also activate different signaling pathways in the cells, leading to the expression of antimicrobial peptides specific for a certain pathogen. In this way PGRP-SA activates the Toll pathway, leading to expression of Drosomycin, which specifically kills Gram-positive bacteria (Gobert et al. 2003), yeast and fungi, while PGRP-LC activates the Imd pathway, which leads to the transcription of Diptericin bactericidal to *E. coli* and other Gram-negative bacteria (Gottar et al. 2002; Choe et al. 2002). In addition to its immune signaling function, PGRP-LC has also been identified as a phagocytosis receptor for Gram-negative *E. coli* on S2 cells: knock down leads to a significant reduction in *E.*

coli engulfment (Rämet et al. 2002). To further investigate the possibility that PGRPs might have functions in phagocytosis, we tested nine members of the PGRP family in our S2 screen (**figure 14, p. 45; figure 15, p. 50**). While we confirmed known functions for *pgrp-sa* and *–lc*, we were very surprised to find that they also seem to be required for the other bacterial type as well: *pgrp-sa* was also necessary for Gram-negative engulfment, and *pgrp-lc* for Gram-positive engulfment. Moreover, knockdown of both genes also seemed to affect apoptotic cell uptake in the cell-based assay (**figure 10, p. 36; figure 14, p. 45**). To see whether these intriguing findings held true *in vivo*, we tested null mutants of both genes, *totem* (*pgrp-lc* -/-) and *semmelweiss* (*seml*, *pgrp-sa* -/-) in our embryonic apoptotic clearance assay as well as in the adult infection assays with *E. coli* and *S. aureus*. As expected, *seml* dies much faster from *S. aureus* infection than control flies, and *totem* flies are susceptible to *E. coli* infection. However, removal of *pgrp-lc* not only increases the flies' susceptibility to *E. coli*, but also to *S. aureus*, and vice versa: *pgrp-sa* flies die significantly faster from *E. coli* as well, demonstrating that each of these factors are required for both Gram-negative and Gram-positive bacteria, which is in line with a recent finding coming to similar conclusions (S. Meister et al. 2009, **figures 19, 20, p. 67, 68**). While it is unexpected but not entirely surprising that PGRPs can be cross-specific for different types of peptidoglycan and therefore promote engulfment of different types of bacteria, we were surprised to see that both genes showed phenotypes in the apoptotic cell assay (**figure 14, p. 45**). For *in vivo* testing we needed to know whether these genes were expressed in the embryo. *pgrp-sa* is expressed in S2 cells, which are derived from embryonic macrophages, but according to three different expression profiles of S2 cells available to us, *pgrp-lc* is not. However, a paper investigating *pgrp-lc* function using RT-PCR shows that this gene is expressed and can be knocked down by specific RNAi (Rämet et al. 2002, **table 2, p. 29**). Also high throughput expression data (Flybase) show that both genes are expressed in the embryo, therefore we presumed that both genes are expressed and proceeded to test the null mutants in the apoptotic cell assay *in vivo*. *pgrp-sa* failed to show any significant effect. In contrast, *pgrp-lc* embryos displayed a twofold increase of apoptotic material in the *Drosophila* embryo, which shows that *pgrp-lc* is also required for phagocytosis of apoptotic cells, confirming the cell-based result (**figure 18, p. 65**). This finding is remarkable because it shows that apoptotic cells and bacteria must share surface determinants. More specifically, an apoptotic cell seems to expose ligands similar to bacterial peptidoglycan on its surface, leading to ligation by PGRP-LC on macrophages and subsequent engulfment of the corpse.

Here we demonstrate for the first time that a peptidoglycan recognition molecule can play a role in the recognition and phagocytosis of apoptotic cells.

Taken together, we were able to identify many novel players in the engulfment process. We found novel factors in glial phagocytosis of apoptotic cells in the *Drosophila* embryo including bridging molecules, surface receptors and intracellular factors. We also found new factors required for phagocytosis of bacteria, which are necessary for the survival of an infection in the adult fly. Surprisingly, many of the factors showed cross-specificity and were also required for apoptotic cell phagocytosis by macrophages in embryonic development. Conversely, some of the apoptotic cell receptors also were cross-specific for bacteria and required for infection survival in the adult fly.

7 Discussion

7.1 Screening for phagocytosis factors in S2 cells

In order to shed more light on the developmentally and immunologically crucial process of phagocytosis, we performed a medium throughput candidate RNAi screen in *Drosophila* S2 cells, followed by *in vivo* validation using transgenic RNAi and mutant flies. Cell-based RNAi screens have been used extensively in *Drosophila* to investigate a multitude of cellular processes including growth and viability, signaling, cell shape, cell division and various other cellular and immune functions including phagocytosis (Cronin et al. 2009; Stroschein-Stevenson et al. 2006; Philips et al. 2005; Agaisse et al. 2005; Gwack et al. 2006; Hao et al. 2008; Dobbelaere et al. 2008; Nybakken et al. 2005; Guo et al. 2008). While the first generation of RNAi libraries produced large numbers of OTE leading to high false positive rates, the next generation libraries, like the one used in this screen (HD2, kindly provided by Michael Boutros) were optimized in that regard, which significantly increased RNAi specificity and validity of results (Mohr et al. 2010; Boutros & Ahringer 2008; Horn et al. 2010; Seinen et al. 2011). Still, overlap between screens remained small, which is attributable to the differences in the techniques and protocols employed, stressing the importance of secondary screening. We therefore, according to current practice and in a first step to exclude false positives, designed secondary non-overlapping dsRNAs for 19% of our candidates to assess the OTE in our assays. Less than a fifth of the secondaries displayed discrepant results, which is on the lower end of what was to be expected from the literature, where discrepancies in secondary RNAs ranged from 15% to 84% (Chew et al. 2009; T. Liu et al. 2009; Wendler et al. 2010, Hao et al. 2008; Sathyanarayanan et al. 2008, Guo et al. 2008). A large majority of the RNAs used were specific for their target genes.

When we look at results from other screens in comparison to ours, it first of all should it be noted that overlap between those screens is already small, with few hits being found in all or even multiple screens, and for the few genes that are found in more than one screen, a large majority has significantly different effect strengths. Where do these discrepancies come from? When we compare the protocols used to conduct those RNAi phagocytosis screens, there are many differences between all studies in all relevant parameters – numbers of cells transfected, amount of dsRNA administered, duration of RNAi treatment, cell-to-food ratio, eating time, and read-

out. Collectively these methodological differences are likely to be the reason for the small overlap between screens. We optimized our protocol for all of the above parameters to achieve a maximum differential in phagocytosis between positive and negative control. Even though individual effect strengths were often significantly different between our results and published data, we still were able to make the same binary calls – involved or not involved - for most of the genes previously tested (24/38 tests = 63% consistency). In summary, our secondary testing and comparisons with other studies raised confidence that we could use hits from this primary screen for further testing.

7.2 Drosophila – a suitable *in vivo* model to study phagocytosis

To really assess a gene's function in a given process, it is however desirable to leave the milieu of cultured cells behind, which is very specific and highly susceptible to environmental changes, and proceed to *in vivo* testing. Genetic invertebrate models are useful for *in vivo* studies because of their short generation time, their relatively small size, simpler body plan and the genetic tools available. The two main invertebrate models are *C. elegans* and *D. melanogaster*, and both have been successful models for phagocytosis studies. In comparison to *C. elegans*, the linked processes of developmental apoptosis and phagocytosis have become much more complex in fly. While cell death is predetermined in the few cells that die during worm development, death during fly development occurs mostly stochastically and in large waves (Rogulja-Ortmann et al. 2007; Lettre & Hengartner 2006). Neighboring cells are sufficient to clear the occasional apoptotic cell in *C. elegans*, but *Drosophila* has specialized cells performing the increased task, namely glia in the nervous system and macrophages everywhere else. In contrast to *C. elegans*, which seems to rely mostly on humoral immunity, *Drosophila* also has an immune system very similar to the innate arm of vertebrate immunity, and professional phagocytes are well equipped to recognize and engulf dangerous invaders as well as initiate signaling cascades, which lead to a systemic immune response (Engelmann & Pujol 2010; Brennan & Anderson 2004). This professionalization of phagocytes is facilitated by a molecular diversification: while *C. elegans* only has the engulfment receptor ced-1, *Drosophila* possesses a whole gene family of related proteins, the NIMs, that seem to be involved in different types of phagocytosis: the ced-1 homolog *drpr* and *simu* are engulfment receptors for apoptotic cells, and NIMC1 is required for bacterial phagocytosis. In the present study we describe new functions for two additional

members of the NIM family. *nimA* is required for apoptotic clearance in the embryo, and *nimC2* for *S. aureus* phagocytosis in the adult fly, thereby firmly establishing the NIMs as a family of diverse phagocytosis receptors.

Another gene family present in fly, but not worm, are the PGRPs. These genes have been shown to play crucial roles in *Drosophila* immunity, because different members are specific for certain types of infections and not only promote engulfment of the respective pathogens, but trigger signaling cascades leading to the expression of pathogen-specific AMPs (Royet & Dziarski 2007). In the present study we revisited some of the players and surprisingly found that *pgrp-lc*, which had been known for initiating immune signaling against an *E. coli* infection, is in fact also required for apoptotic clearance in the *Drosophila* embryo, thereby expanding its function as an immune receptor.

Drosophila possesses not only diversified families of genes present in the worm, it also contains homologs to vertebrate phagocytosis factors. Vertebrate *cd36* and *megalin* have been linked to apoptotic clearance and *Drosophila* has homologs of these genes, which are expressed in macrophages and glia. While the cd36 homolog *crq* has already been shown to be required for apoptotic clearance by macrophages in the *Drosophila* embryo, we show here that an additional homolog, *santa maria*, is required for glial clearance of apoptotic cells. Also *megalin*, which promotes phagocytosis of apoptotic cells in vertebrate macrophages, is expressed in *Drosophila* glia and, as we show here, is necessary for engulfment. We examine some additional factors that have been shown to play a role in vertebrates and find ubiquitous factors like Annexin and Calreticulin, which have been involved in apoptotic clearance in vertebrates, to be highly expressed in *Drosophila* glia and also required for apoptotic clearance here, underlining the similarities between phagocytosis in the fly and in vertebrates.

While the sophistication of the molecular repertoire of *Drosophila* innate immune cells, remiscent of vertebrate macrophages, is remarkable, the real immunological achievement in vertebrates is the adaptive immune system. Vertebrates somatically rearrange immune receptors producing an infinite number of recognition molecules able to recognize every possible pathogen. Invertebrates are believed to not possess such a system; however it has been shown that the cell adhesion molecule DSCAM is produced in thousands of different splice isoforms, which recognize Gram-negative infections and have even been shown to be individually upregulated in

response to specific pathogens (Watson et al. 2005; Dong et al. 2006). Moreover, we found DSCAM to also have another function, namely apoptotic clearance in the developing embryo. The diversity of DSCAM isoforms and the NIM and PGRP families place the *Drosophila* immune system and its phagocytes in between the worm and vertebrate models and make it an excellent model to study phagocytosis and immune functions.

7.2.1 Dissecting phagocytosis *in vivo*

To check whether our cell-based results track *in vivo*, we selected a subset of genes from the primary screen that were of particular interest to us (18 genes = 21% of all genes tested), on the basis that they were either highly expressed in glia, which is our primary focus, or showed new or unexpected food preferences. Many of these genes had been described in contexts other than phagocytosis, or had even been implicated in phagocytosis, but only of one specific food type, and we wanted to test its involvement in other types of phagocytosis. For four out of the 18 genes we were able to use available null mutants for our *in vivo* testing, for the others we used transgenic RNAi lines, which we expressed in macrophages or glia. We picked not only receptors, but chose genes to go along a longitudinal axis through the cell, from secreted and transmembrane receptors to the core machinery with signaling and vesicle traffic factors. We had seen that some receptors, previously claimed to be specific for apoptotic cells or a certain bacterial type, in fact seemed to be required for other types of food as well, based on our cell-based results. These intriguing findings led us to also cross-compare *in vivo* the different food types and not only focus on developmental clearance of corpses, but also investigate immunological clearance of pathogens. Therefore we tested all candidates in three previously established *in vivo* assays: apoptotic clearance in the embryo, and infection of the adult fly with *E. coli* or *S. aureus*. Even though phagocytosis of endogenous dying cells and of invaders is accomplished by the same cells and arguably using the same mechanisms, no systematic comparison between corpse and bacterial eating has been carried out to date; the fields of developmental genetics and immunology seem to not touch and our *in vitro* and *in vivo* comparison of these different yet similar processes is the first of its kind.

7.2.2 *In vitro* results track *in vivo*

Out of the 40 *in vivo* assays performed, a vast majority (93%) were congruent with our cell-based results, which is remarkably high given the different nature of a cell

culture flask compared to a whole fly. Because we were mostly working with RNAi, some of our results could be false positives or false negatives. We used *in vitro* and also largely *in vivo* RNAi to knock down gene function, thereby creating hypomorphs. The obtained reduction in gene expression may not be sufficient leading to false negatives. In contrast, RNAi, despite optimizations in RNAi design, is known to have OTE, which could knock down additional genes thereby creating false positives. However, our combined approach of cell-based and *in vivo* testing alleviates these issues because the cell-based and *in vivo* data provide excellent controls for each other. Effects observed in both systems, which are fundamentally different from each other (cell line vs. whole animal, transfected vs. transgenic RNAi, different dsRNA sequences), cumulatively raise confidence that an observed phenotype is significant. Also the overall satisfactory consistency between cell-based and *in vivo* data shows that our approach proves valid to identify new phagocytosis factors.

It was particularly interesting to see that the apoptotic assay tracked exceptionally well *in vivo* (17 out of 19 genes tested), even though for some genes we tested macrophages *in vitro* and glia *in vivo*, corroborating the similarities between these cell types. There are two cases in the apoptotic cell assay where *in vitro* and *in vivo* did not match up: 1. *pgrp-sa*, were we saw an *in vitro*, but not an *in vivo* effect using a null mutant, which is potentially due to compensatory mechanisms *in vivo*; 2. For *skittles*, a PI3P kinase, we observed an effect in cells but not using transgenic RNAi *in vivo*, which could be explained either by an OTE in cells or insufficient RNAi knock down *in vivo*. Our *S. aureus* assays were similarly consistent with the cell-based results; here only one gene was discrepant. The biggest issue in reproducibility of cell-based results posed the *E. coli* assay. In contrast to *S. aureus*, *E. coli* is not pathogenic to wild type flies. Only few genes have been described to have any effect on the flies' susceptibility to *E. coli* infection, and all of them are components of the IMD signaling pathway leading to expression of antibacterial peptides (Pal & Louisa P Wu 2009). Consequently, when we infect *pgrp-lc* – the Imd pathway receptor – flies with *E. coli*, half of the cohort is dead after two days. However, when we infect flies where *pgrp-lc* has been knocked down in macrophages using transgenic RNAi, we see no phenotypes. Similarly, when we tested our candidates, we saw effects for the null mutants, but not for any of the RNAi lines, suggesting that *E. coli*'s low pathogenicity combined with insufficient gene knock downs failed to reduce protein amounts to phenocritical levels. Another possible explanation, especially for the genes upregulated in glia, would be that these genes are not (highly) expressed and therefore less relevant in adult macrophages. Remarkably, while RNAi has been

used in the literature to test different genes' involvement in Gram-negative infections, there has not been a report so far about RNAi of any gene reducing *Drosophila*'s survival to an *E. coli* infection (Bischoff et al. 2006; Kambris et al. 2006; Sebastien Pili-Floury et al. 2004; Zaidman-Rémy et al. 2006). For two secreted genes, namely the thioester-containing complement genes *tep2* and *mcr*, we saw an effect *in vitro*, but not *in vivo*. While this could be also attributed to the aforementioned issues, this is consistent with the literature, suggesting these genes play a more important role in embryonic macrophages than in adult ones. Because of the weakness of transgenic RNAi in *E. coli* infection we only included the results obtained from infecting null mutants with *E. coli* in our analysis. For the four genes for which we had null mutants available, we were able to reproduce the cell-based results *in vivo,* thereby identifying new players in Gram-negative phagocytosis.

7.2.3 *In vivo* results track with literature

Most of our candidates were not entirely novel genes without any known function, but rather factors that had been implicated more or less well in one or another functional process, some in phagocytosis of a different food type. Therefore it was an important proof of principle to be able to recover known functions with our methodology. When we compare our *in vivo* findings with those from other studies, we find that all of our observations are consistent with published results. This, in contrast to the lower overlap between cell-based screens, underlines the importance of *in vivo* testing, which is apparently highly robust across different research groups and protocols.

7.3 Novel glial players in corpse removal

Our screen was initially motivated to elucidate the relatively recent discovery of *Drosophila* glia as competent and almost professional cells that can efficiently remove dying neurons in the developing CNS; in *simu* we found the second factor ever identified that is required for glial corpse clearance (Kurant et al. 2008). The expression profiling of embryonic glia performed in our lab, which also produced *simu*, showed that multiple putative phagocytosis genes were ranking high in the list, supporting the idea of glia as semi-professional phagocytes, and providing us with interesting candidates to further explore glial function.

calreticulin is an example of a ubiquitously expressed chaperone. The function of its upregulation in glia might have been elusive had there not been reports about *crc*

playing a role in apoptotic clearance in mouse macrophages (Michalak et al. 2009; Gardai et al. 2005; Vandivier et al. 2002; Müller-Taubenberger et al. 2001). Our main goal was not comparing macrophages with glia, but exploring glial function. Because *crc* is highly upregulated in glia we decided to see whether it plays any role in corpse removal by glia in the embryonic CNS. Indeed we saw a requirement for *crc* in glia for clearance of apoptotic cells, which is in line with previous reports that CRC can act as a (co-)receptor for apoptotic cells in vertebrates (Vandivier et al. 2002). Another conserved player highly expressed in glia is *annexinIX*, removal of which has been shown to be involved in apoptotic clearance in worm, and inflammation and autoimmune disease in mouse (Arur et al. 2003; Y. H. Yang et al. 2004; Hannon et al. 2003). ANNIX is a secreted molecule and acts as an opsonin by recognizing newly exposed PS on the apoptotic surface, and we observe that also in *Drosophila,* knock down of *annIX* leads to impairment of apoptotic clearance, probably because the apoptotic cells lose an important “eat-me” signal. This hypothesis is corroborated by the fact that injection of fluorescent recombinant ANNV into the *Drosophila* embryo specifically labels early apoptotic cells in the nervous system (Kurant et al. 2008). It seems that glia produce, secrete and eventually recognize apoptotic cells using ANNIX; the last step however, the ligation of ANNIX-opsonized apoptotic cells to the glia, requires a yet to be identified receptor on the glial surface. In vertebrates it has been suggested that ANN coclusters with PS on the apoptotic cell surface, thereby facilitating binding of the complex by a PS-receptor on the phagocyte (Arur et al. 2003). However the identified PS-receptor turned out to be a transcription factor, mystifying its role in the process (Cui et al. 2004; Mitchell et al. 2006; Williamson & Schlegel 2004).

CD36 and LDLR family members are classical scavenger receptors with broad ligand specificity and have been known to be expressed in macrophages, where they facilitate disposal of hydrophobic material including lipids and lipoproteins from body fluids. Here we report involvement of two *Drosophila* homologs belonging to these families in glial clearance of apoptotic cells.

The CD36 family of lipoprotein-binding receptors is evolutionarily conserved and is required for lipoprotein transport and uptake of cholesterol and lipids in vertebrates (Collot-Teixeira et al. 2007). CD36 has been associated with a variety of human disorders including insulin resistance, dyslipidemia, and atherosclerosis. Intriguingly, CD36 and its homologs have been implicated in various types of phagocytosis in vertebrates, worm and fly (Franc, White, et al. 1999). CD36 family members are

highly conserved, and the CD36 homolog *crq* in *Drosophila* has also been shown to be required for phagocytosis of apoptotic cells, in this case by macrophages in the developing embryo (Franc, Heitzler, et al. 1999). In the glial expression profiling from our lab a gene very similar to *crq* was shown to be highly expressed in embryonic glia: *santa-maria*, a gene that had been previously involved in fatty acid uptake by neurons and glia in the adult eye (Wang et al. 2007). Intriguingly, we found *santa maria* to be required for glial removal of corpses in the embryonic CNS. How does *santa maria* recognize apoptotic cells in the CNS? It has been shown in vertebrate cell-culture experiments that vesicles containing oxidized (but not nonoxidized) PS inhibit phagocytosis of apoptotic cells in a CD36-dependent manner (Greenberg et al. 2006). Although little is known about the ligands promoting apoptotic cell phagocytosis in *Drosophila*, we have previously shown that PS is likely to play a role here, as PS-binding Annexin labels apoptotic cells in the embryonic CNS, therefore it is possible that SANTA MARIA's ligand on apoptotic cells is oxPS (Kurant et al. 2008).

Another putatively lipid-binding scavenger receptor is *megalin. megalin is* a large multiligand receptor and member of the LDL receptor family. It is widely expressed in epithelial tissues and mediates endocytosis of a variety of ligands from the apical surface (Christensen & Willnow 1999; Moestrup & Verroust 2001). In *Drosophila*, *megalin* has recently been shown to be required for internalization of the pigment protein *yellow*, thereby regulating cuticle pigmentation in the adult wing (Riedel et al. 2011). *megalin* is highly expressed in embryonic glia, according to our expression profiling, and interestingly shares some sequence similarity in its intracellular portion to *drpr*. As we show here, *megalin* is necessary for glial corpse clearance. Members of the vertebrate LDLR family have also previously been implicated in phagocytosis: LDLR in phagocytosis of aggregated oxidized LDL, and LDLR related protein (LRP) in phagocytosis of aggregated LDL as well as phagocytosis of apoptotic cells. Members of the LDLR family have wide ligand specificity and recognize various lipids and lipoproteins. Of particular interest are oxLDL and PS, which are present on apoptotic cells. It has been shown that blocking both lipids inhibits phagocytosis of apoptotic cells, suggesting that these are at least some of the ligands recognized by *Drosophila* glia on apoptotic cells (Sambrano & Steinberg 1995).

We found not only that putative apoptotic cell receptors are required for glial phagocytic function, but curiously a protease normally required for ECM degradation also plays a role in the process, *mmp1*. *mmp's* are highly conserved and have been

implicated in a variety of tissue remodeling processes including bone formation and remodeling, mammary development, blood vessel formation, inflammation and wound healing (Page-McCaw et al. 2007). In *Drosophila* it has been shown that null mutants for either of the two fly *mmp's* leads to aberrant axon defasciculation in the embryo, perturbing normal nervous system development, as well as causing abnormal tracheal development (C. M. Miller et al. 2008; Glasheen et al. 2010; Yasunaga et al. 2010). These functions are accomplished by proteolytic cleavage of a variety of substrates including ECM components, cell-surface receptors and cyto- and chemokines, thereby changing tissue composition and properties. The only hint at a function in phagocytosis stems from reports stating that a mouse homolog of *mmp1*, Mt-1 MMP, is implicated in degrading collagen fibrils to prepare them for phagocytic uptake by macrophages, a process relevant to wound healing, inflammatory diseases, and cancer cell invasion (H. Lee et al. 2007). *mmp1* was highly ranked in the glial expression profile and our *in vivo* knockdown in glia significantly reduces glial phagocytosis of apoptotic cells in the developing CNS, for the first time establishing a member of this group of proteases in the engulfment process. How MMP1 assists glia in engulfing apoptotic cells remains to be elucidated; based on the many possible functional mechanisms, it is conceivable that secreted MMP1 shears a "don't eat me signal" from the apoptotic cell, or, reversely, activates an "eat-me" or "find-me" signal. Another explanation could be that it simply helps glia in probing their surroundings by shearing away matrix components in the immediate vicinity of the phagocytosing glia.

As our goal was not only want to find new surface receptors specific for apoptotic cells, which, given the systematically unspecific nature of many receptors, turns out to be challenging, downstream effectors from vesicle trafficking were also included. We had assumed a broad and unspecific requirement for these factors in any type of phagocytosis, but at least for the two candidates picked for *in vivo* validation, obtained the opposite result. SYB and Cyp4g15, loosely appointed to vesicle traffic based on their subcellular localizations – exosomes for SYB and ER for the Cytochrome P450 family member Cyp4g15, both showed effects exclusively in apoptotic clearance. *syb* also showed a requirement for both types of bacterial phagocytosis in our cell-based assays, but not *in vivo*. This discrepancy might be explained by the nature of the *syb* function, which had been elucidated in mouse cell culture studies. *syb* is a membrane fusion protein localized on late endosomes, and when phagocytes extend pseudopods to enwrap their target, *syb* is required for rapid membrane delivery to the phagocytic cup through exocytosis of these late

endosomes (Braun et al. 2004). It seems logical that *syb* requirement would depend on the target particle's size. In the cell-culture assay, bacteria-to-phagocyte-ratios are high in order to achieve homogenous coverage of cells with food. Therefore engulfment of large apoptotic cells or chunks of (dead) bacteria *in vitro* requires more membrane delivered to the phagocytic cup than engulfment of small live bacteria that are solitary, which is more likely to be the case *in vivo*. Another explanation is of course a differential requirement for *syb* in macrophages. We knocked down *syb* in macrophages, and not in glia for the adult infection assays. Macrophages might be able compensate for lack of *syb*, while glia could not.

It seems that glia are fully equipped for the task of apoptotic clearance in a variety of ways that this study helped to unveil. Glia express a multitude of specific and unspecific apoptosis receptors on their surface and even secrete MMP1 and opsonins into the extracellular space to trace nascent apoptotic cells. They upregulate vesicle traffic constituents in order to be prepared to quickly engulf and clear away big corpses. *Drosophila* glia, even though stationary and not motile like macrophages, have a large battery of tools that allow them to detect, engulf and degrade apoptotic cells as efficiently as the real professionals – macrophages, calling for glia to be classified as quasi-professional phagocytes.

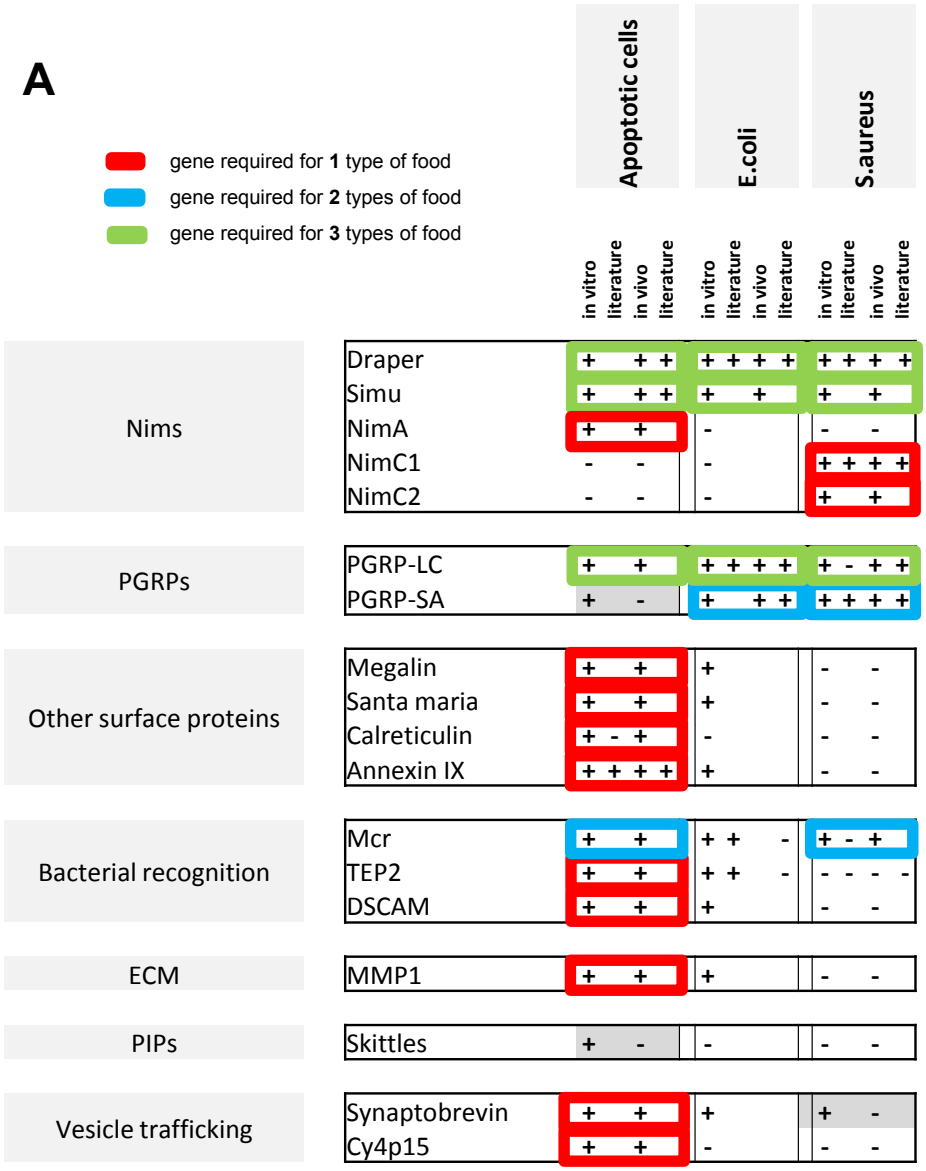
7.4 Cross-specificity

One of the key findings in our screen was that many genes are not only required for phagocytosis of one type of food, but actually for multiple food types. This systematically reveals for the first time in *Drosophila* that there is overlap between apoptotic and bacterial clearance. In fact, in the cell-based screen only 20% of genes were specific to one food type, and most of those for apoptotic cells (**figure 16, p. 54**). Even though we tested only a relatively small set of genes, there is no inherent bias in the selection: of the genes chosen, seven had previously been implicated in corpse clearance, and eight in bacterial clearance. Interestingly, the cross-specificity is not highest for the two bacteria tested, but for apoptotic cells and one or even both types of bacteria. This is surprising, as foreign prokaryotic bacteria seem to be fundamentally different from self-derived apoptotic cells, yet apparently macrophage factors promoting phagocytosis do not distinguish that much between the food types presented. When we look at the *in vivo* results, this overlap in food specificity continues (**figure 21, p. 84**): macrophage receptors in particular are exceptionally promiscuous in their food choice: five out of the ten tested macrophage receptors

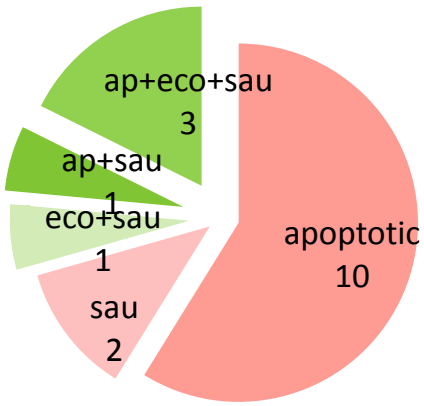
recognize not just one specific food type, but two or even all three of the tested targets. Again, specificity is crossed not only between Gram-negative and Gram-positive bacteria, but actually more often between bacteria and apoptotic cells (four genes).

Which type of genes do the promiscuous factors belong to? SIMU and DRPR were the anchor points for phagocytosis research in our lab and led to extensive studies of their functions in apoptotic clearance in the developing embryo. Both genes act in conjunction in the same pathway for corpse removal, and knockout of either of them leads to an accumulation of apoptotic material in the embryo (Kurant et al. 2008). Recently both genes have been identified as members of a protein family of related genes, the NIM family. Some members of the NIMs are required for bacterial phagocytosis: *nimC1* for *S. aureus* (Kurucz et al. 2007) and *eater* for both *S. aureus* and *E. coli* (Kocks et al. 2005). Also *drpr* was recently shown to play a role in clearance of *E. coli* and *S. aureus* in addition to its known role in corpse clearance (Cuttell et al. 2008).

A


gene required for 1 type of food
gene required for 2 types of food
gene required for 3 types of food
Apoptotic cells
E.coli
S.aureus
in vitro literature
in vivo literature
in vitro literature
in vivo literature
in vitro literature
in vivo literature
Nims
Draper + + + + + + + + + + + +
Simu + + + + + + + +
NimA + + - -
NimC1 - - - + + + +
NimC2 - - - + +
PGRPs
PGRP-LC + + + + + + + - + +
PGRP-SA + - + + + + + + + +
Other surface proteins
Megalin + + + - -
Santa maria + + + - -
Calreticulin + - + - - -
Annexin IX + + + + + - -
Bacterial recognition
Mcr + + + + - + - +
TEP2 + + + + - - - - -
DSCAM + + + - -
ECM
MMP1 + + + - -
PIPs
Skittles + - - - -
Vesicle trafficking
Synaptobrevin + + + + -
Cy4p15 + + - - -

B


ap+eco+sau
3
ap+sau
1
eco+sau
1
sau
2
apoptotic
10

Previous page:

Figure 21: Cross-specificity of phagocytosis genes tested in vivo.

A *Shown is presence (+) or absence (-) of an effect on phagocytosis of apoptotic cells,* E. coli *and* S. aureus *in the cell-based* in vitro *screen as well as* in vivo*. Many genes are required for phagocytosis of multiple food types. Genes required for 1 type of food are in red, for 2 types of food in blue and for 3 types of food in green boxes. Shaded in grey are results that are discrepant between cell-based and* in vivo *testing in our hands.* ***B*** *Pie chart displaying gene proportions affecting single food types or combinations of foods in the* in vivo *assays. Displayed are only genes that have been validated* in vivo*. The reds show proportions of factors specific to only one type of food. Light green: genes affecting 2 types of food. Bright green, 3 genes showed phenotypes in all three* in vivo *assays.*

Still, we were very surprised to find *drpr* and *simu* to be required not only for apoptotic clearance, nor for just one but for both types of bacterial eating and survival of Gram- positive and Gram-negative infections, turning these genes from specific apoptotic cell receptors to general phagocytosis receptors with a broad target specificity. Our lab has previously shown that SIMU's function does not depend on the presence of its transmembrane domain, but rather on the N-terminal EMI domain, which is likely to specifically interact and recognize molecular patterns on the target surface (Kurant et al. 2008). It will be interesting to see what the EMI-recognized patterns on apoptotic cells and bacteria are, and how they both can elicit SIMU-mediated engulfment.

While *simu* and *drpr* do not seem to distinguish between apoptotic cells, Gram-negative and Gram-positive bacteria, the NIM family also seems to contain members that are specific for certain food types. NIMC1 had already been shown to only recognize *S. aureus*, which we confirm with our *in vitro* and novel *in vivo* results, and additionally find that the closely related *nimC2* also shows the same phenotype. *nimC1* and *nimC2* are structurally similar and have a similar length compared to the other nimrods, which are either much longer or shorter; *nimC1* has 16, and *nimC2* 10 NIM-repeats and both are transmembrane molecules. Despite their similarities they apparently cannot substitute for each other, as both are individually required to fight an aggressive *S. aureus* infection. Even though much more intensely and longer studied, only few phagocytosis receptors are known in the *Drosophila* immune system – a stark contrast to the plethora of identified vertebrate factors (admittedly mostly in cell culture models), and few of these have been tested *in vivo* in the fly

(Stuart & Ezekowitz 2008). Very few studies (Kocks et al. 2005; Gottar et al. 2002; Choe et al. 2002; Bou Aoun et al. 2011; Nehme et al. 2011) actually test their factors in adult fly infection models questioning the relevance of a given factor for fly immunity. While *in vivo* assays like thoracal injection of fluorescent bacteria into flies (Cuttell et al. 2008) might be a more direct measure of phagocytosis, monitoring fly survival gives an exact read out of the impact a gene has on the immunity or susceptibility to an infection. Therefore, our confirmation of *nimC1* and discovery of *nimC2*, but also the identification of the less specific *simu*, *drpr* and *mcr* as novel *S. aureus* phagocytosis receptors *in vivo* and the fact that these genes are required to longer withstand an infection in the adult fly provide an important increase of knowledge for the field of fly immunity.

While *drpr* turned out to be quite a promiscuous player, the most closely related NIM is not: we find *nimA* to be exclusively required for phagocytosis of apoptotic cells. It remains to be tested whether *nimA* functions in the same pathway as *simu* and *drpr* and acts as an interchangeable *drpr* homolog, or whether it functions independently of this pathway. Its exact function in phagocytosis remains to be elucidated, but its molecular structure, which is so similar to DRPR suggests that NIMA is also a recognition receptor for apoptotic cells. *nimA*, like *drpr*, has an N-terminal EMI-domain followed by exactly one NIM repeat and two (instead of 15) EGF-like repeats making it the only other family member to contain EGF-like repeats. Moreover, *nimA* and *drpr* are the only two family members to contain a large intracellular domain, which in the case of DRPR has been shown to promote signaling though activation of the non-receptor tyrosine kinase SHARK by binding its intracellular src-phosphorylation motif (Ziegenfuss et al. 2008). *nimA*'s exclusivity for embryonic corpse clearance is not due to a lack of expression in the adult fly, as RT-PCR analyses show the gene to be expressed throughout all developmental stages and in adulthood. Therefore it will be interesting to study what it is that allows DRPR, but not NIMA, to recognize bacteria in addition to apoptotic cells.

Another protein class of phagocytosis factors are the PGRPs, and for the first time we report that the *E. coli* receptor PGRP-LC, arguably the most studied gene in the whole family, is not only not specific for *E. coli*, which had been already suggested by one study (S. Meister et al. 2009), but not even specific to bacteria, as it is strongly required for apoptotic clearance in the *Drosophila* embryo. This is a slightly different case than for *simu* and *drpr*, because PGRP-LC is thought to activate the IMD pathway. This initiates an immune signaling cascade leading to the activation of

Drosophila's humoral arm of defense, namely the expression of antibacterial peptides, which are released into the hemolymph and specifically destroy Gram-negative bacteria (Royet & Dziarski 2007). Phagocytosis of apoptotic cells, however, is supposed to be an immunologically silent event: in higher organisms, which are in danger of acquiring auto-immune diseases due to adaptive immunity, macrophages, after engulfing an apoptotic cell, even release anti-inflammatory cytokines to avoid inflammation (Krysko et al. 2006). In *Drosophila* we at least know that the IMD pathway is not activated unless there is a real threat – a Gram-negative infection – which leads us to the question: how can the same receptor on the one hand promote proinflammatory signaling when recognizing a specific bacteria, and on the other hand facilitate only silent engulfment, when faced with a dying cell? One answer could be that *Drosophila* relies on combinatorial input with other receptors, similar to the successful vertebrate model, where recognition of pathogen-associated molecular patterns is outsourced to the toll-like receptors, a non-phagocytosing receptor class exclusively dedicated to pathogen recognition (Takeda et al. 2003). According to this model PGRP-LC promotes engulfment in any case, but activates immune signaling only in conjunction with a putative second *E. coli* receptor, which cannot facilitate phagocytosis itself.

The third family of putative phagocytosis receptors, members of which we included in our screen, are the TEPs. This gene family is highly conserved in evolution and its function is to opsonize their bacterial targets for phagocytosis or, at least in vertebrates, instant lysis (Philippe 2004; Blandin & Levashina 2004). *Drosophila* has five functional *tep* genes, four of which we included in our screen and two of which were tested *in vivo*. *mcr* as well as *tep2* were identified in a S2 cell screen as bacterial receptors for both *S. aureus* and *E. coli*, and we recover these known functions in our S2 screen (Stroschein-Stevenson et al. 2006). A recent study tested *tep* involvement in survival of different Gram-positive and -negative infections as well as phagocytosis *in vivo* using tep mutants, in which gene expression is strongly reduced due to P-element insertions at the transcription start sites or, for *tep1*, ubiquitously expressed transgenic RNAi (Bou Aoun et al. 2011; Thibault et al. 2004). Surprisingly, the authors could not find any requirement for any of the genes in any infection, suggesting that these genes can substitute for each other *in vivo*. However, even double and triple mutants did not succumb faster to infections than control flies. The one gene not tested in this study was *mcr*, because the mutant is larval lethal. *mcr* was identified in a S2 screen for fungal phagocytosis, but in our S2 screen we find it also to be required for *S. aureus* phagocytosis. Our *in vivo* data support that

finding and show that knockdown of *mcr* in macrophages reduces fly survival of *S. aureus* infection, thereby for the first time revealing a function for any of the TEP family members *in vivo* in *Drosophila*.

7.5 Apoptotic cell opsonins

Both *tep2* and *mcr* were unexpectedly required for apoptotic cell phagocytosis according to our cell-based screen, and these results were confirmed *in vivo*, where both genes were required for corpse clearance in the embryo by macrophages. TEP involvement in apoptotic clearance is interesting not only from the standpoint of cross-specificity, but also in that this, along with the earlier described glial annIX, is the first time secreted factors are being implicated in apoptotic cell clearance in *Drosophila*. The mechanism of opsonization in general is the release of opsonins into the body fluid, recognition of and attachment to molecular patterns – classically, specific bacterial sugar residues – on target particles (opsonization itself), and recognition of these closely clustered surface “labels” by specific receptors on phagocytes. The concept of opsonins acting not only on pathogens, but also on apoptotic cells is not new; a number of so-called bridging molecules recognizing altered plasma membrane lipids and proteins have been described mainly in vertebrates, but also *C. elegans*, the most prominent being AnnexinI/V, which recognizes freshly exposed phosphatidylserine on apoptotic cells. Interestingly, most factors that showed apoptotic clearance phenotypes *in vivo* in vertebrates were not macrophage receptors, but such serum opsonins/ bridging molecules (Y. Wu et al. 2006). These factors are transported by the blood and lymph through all body tissues and therefore act as sensors of apoptotic events, and when those bridging molecules recognize a corpse, phagocytes are recruited for engulfment. It seems that this early warning system is particularly sensitive to perturbations, and single gene knockouts in mice already lead to severe autoimmune diseases. In *Drosophila*, however, such factors had not been described to date. While for the vertebrate complement proteins, the macrophage receptors have been identified (Philippe 2004), it is not clear how bridging molecules are ligated to phagocytes, and also how TEP-opsonized apoptotic cells are recognized and engulfed by macrophages in *Drosophila* remains to be elucidated.

Another secreted molecule we found to unexpectedly play a role in corpse clearance is DSCAM, an Immunoglobulin superfamily member that had been initially described in the context of neuronal wiring (Hattori et al. 2007). Due to ca 18.000 splice

isoforms that are generated by alternative splicing, these molecules are able to confer highly specific cell-cell adhesion through homophilic binding of identical receptor isoforms on different cells. The large number of possible isoforms gives cells distinct identities and thereby facilitates correct connectivity between neurons (Schmucker & B. Chen 2009). Recently it has been found that DSCAM is also expressed by hemocytes, which produce a similar variety of splice isoforms, but then, at least partially, secrete the immunoglobulins into the hemolymph. S2 cell phagocytosis of *E. coli* could be blocked by anti-DSCAM antibodies and *dscam* expression was shown to be necessary for *E. coli* phagocytosis by larval hemocytes; furthermore specific DSCAM isoforms were shown to bind *E. coli* (Watson et al. 2005). Spectacularly, it has been found that in mosquitoes, hemocytes' *dscam* expression is not random, but depends on the infection and that specific splice isoforms are upregulated in response to specific pathogens, reminiscent of clonal selection of antibodies – also immunoglobulins – and T-cell receptors during vertebrate adaptive immune responses (Dong et al. 2006). We included *dscam* in our screen based on the possibility of it being a phagocytosis receptor, and indeed observed a requirement for *E. coli* phagocytosis by S2 cells as had previously been described. Surprisingly, we also saw *dscam* involvement in apoptotic clearance in S2 cells and were able to validate this phenotype *in vivo*, where it was required for corpse clearance in the embryo by macrophages. This finding suggests a completely novel role for this factor in addition to neuronal wiring and immunity. How DSCAM functions in recognition and uptake of corpses is unclear, but could be accomplished through a two-step process: secreted DSCAM isoforms recognize and opsonize apoptotic cells and then homophilically bind to other DSCAM molecules that remained transmembrane on the macrophage surface. Such a mechanism would be similar to the process described in vertebrates, where antibodies recognize oxidized lipids on apoptotic cell surfaces and subsequently promote macrophage engulfment through Fc-receptor ligation (Ogden & Elkon 2006; Hart et al. 2004).

We present here for the first time a systematic study addressing various open questions in phagocytosis research: how do macrophages engulf dying cells during development? How do glia engulf dying neurons during development? What are secreted, surface or downstream factors required for phagocytosis? How do macrophages in the adult fly fight different bacterial infections? What are the overlaps between apoptotic and bacterial clearance? Our comprehensive cell-based and *in vivo* approach shed light on these topics and revealed many new players in this complex process that is crucial for organismal development and homeostasis.

8 Materials and Methods

8.1 Cell culture and RNA bathing

Schneider cells (S2) were maintained in Schneider's *Drosophila* medium (BioSell) supplemented with 10% heat-inactivated FCS (Gibco BRL) and 1% penicillin/ streptomycin (Gibco BRL). For RNA bathing 0.4 x 10^6 cells/ well were plated in serum-free media in 96 well plates, 0.7 µg dsRNA was added, cells were incubated for 45 min and 2 volumes of full media were added. Phagocytosis assays were performed after 72 h.

8.2 Phagocytosis assays

RNAi treated S2 cells were fed different particles: carboxylated yellow-green beads (Polysciences), *E. coli*-AF488, *S. aureus*-AF488 (Invitrogen) or apoptotic cells. Apoptotic cells were generated by adding 75 µg/ ml etoposide to S2 cells for 16 h. Apoptotic cells were fluorescently labeled by incubation with 5 µl/ml DiI (Invitrogen) for 25 min at 37°C and washing twice with PBS. Beads, bacteria or apoptotic cells were added to S2 cells in 50 µl but at different concentrations reflecting different optimized eater:food ratios: 3-5 beads/ cell, ca. 15 bacteria/ cell and 2-3 apoptotic cells/ cell. Cells were spun down to synchronize the start of engulfment and incubated for 2 h (beads), 2 h (*S. aureus*), 2.5 h (*E. coli*) and 3.5 h (apoptotic cells). Extracellular beads and bacteria were quenched using TrypanBlue 1:2 (pH 5.5, Invitrogen).

8.3 Flow cytometry analysis of phagocytosis

FACS Calibur with CellQuest software (BD Biosciences) was used to acquire 5000 S2 cells and record the parameters forward and side scatter, red or green fluorescence and time for each cell. Phagocytosis was assessed using FCS Express (De Novo Software) and calculating the product of percentage and fluorescence mean of positive cells, as has been described before (Rämet et al. 2002; Philips et al. 2005), see also **figure 9A-C, p. 34**. For apoptotic cells the fluorescence mean of the whole eater population was determined as a measure of the amount of engulfed corpses (**figure 9D**). Results were normalized to *gfp* RNA treated controls and experiments were repeated 3-7 times (technical and biological replicates). Means

were calculated and significance was assessed using calculating q-values and false discovery rate analyses.

8.4 Fly strains

The fly strains used in this study were obtained from the following sources:

Table 4: Fly strains used in this study.

***A** miscellaneous sources, **B** VDRC lines*

A

Genotype	Source
w; DD1; PGRP-SA (seml)	M. Ramet
w; PGRP-LCΔ5 (totem)	M. Ramet
drpr Δ5	M. Freeman
simu	E. Kurant
w; P{UAS-dicer2, w[+]}	VDRC
repo-Gal4	V. Auld
crq-Gal4	P. Martin; gift from H. Agaisse and N. Perrimon

B used VDRC RNAi lines

Gene	Transformant ID	RNAi library
AnnexinIX	106867	KK
Calreticulin	51272	GD
Santa maria	33153	GD
Cyp4g15	8034	GD
DSCAM	108835	KK
MEGALIN	105387	KK
Mcr	100197	KK
Mdr65	9019	GD
MMP1	101505	KK
NIMA	104204	KK
NIMC1	105799	KK
NimC2	36264	GD
NimC2	3705	GD
Skittles	101624	KK
Synaptobrevin	102922	KK

TEP2	106997	KK

8.5 dsRNA library

DNA templates and dsRNAs for the 86 candidate genes were obtained from Michael Boutros, DKFZ, Heidelberg. Exact positions and sequences of probes can be viewed here: http://genomernai.de/GenomeRNAi/

Gene name	Fly base ID	RNA probe ID
Annexin IX	CG5730	BKN30217
Arp66B	CG7558	BKN22344
Calreticulin	CG9429	BKN21487
Cdc42	CG12530	BKN28698
CG10702	CG10702	BKN23880
CG7447	CG7447	BKN22763
Coracle	CG11949	BKN29444
CP-1	CG6692	BKN27765
Crk	CG1587	BKN28690
Croquemort	CG4280	BKN23364
Santa maria	CG12789	BKN23760
Cyp4g15	CG11715	BKN25252
dCED-12	CG5336	BKN22884
dCED-6	CG11804	BKN22637
Dmoesin	CG10701	BKN28480
DRPR	CG2086	BKN28333
Dscam	CG17800	BKN20392
dSR-CI	CG4099	BKN23236
Dystroglycan	CG18250	BKN20924
EcR	CG1765	BKN31288
EGFR	CG10079	BKN20147
Eiger	CG12919	BKN23624
Eip63E	CG10579	BKN28615
Fps85D	CG8874	BKN22088
Gartenzwerg	CG8487	BKN20511
Gliolectin	CG6575	BKN30169
Hem/ Kette	CG5837	BKN20432
Hemese	CG31770	BKN30432
Hemomucin	CG3373	BKN27462
inflated	CG9623	BKN28544
Jra	CG2275	BKN20678
MEGALIN	CG34352	BKN20531
Malvolio	CG3671	BKN28406

Gene name	Fly base ID	RNA probe ID
NimB4	CG33115	BKN31092
NimB5	CG16873	HFA 973165
NIMC1	CG8942	BKN30957
NimC2	CG18146	BKN27282
NimC3	CG16880	BKN32612
NimC4 (SIMU)	CG16876	BKN30227
PGRP-LA	CG32042	BKN31407
PGRP-LC	CG4432	BKN24423
PGRP-LE	CG8995	BKN25992
PGRP-LF	CG4437	BKN22639
PGRP-SA	CG11709	BKN27520
PGRP-SB1	CG9681	BKN24293
PGRP-SB2	CG9697	BKN32145
PGRP-SC1A	CG14746	HFA 973177
PGRP-SD	CG7496	BKN25597
Pi3K59F	CG5373	BKN27602
Pi3K68D	CG11621	BKN27399
Pi3K92E	CG4141	BKN22357
Psr	CG5383	BKN27474
PTEN	CG5671	BKN29278
Puckered	CG7850	BKN25001
Pvr	CG8222	BKN21626
Rac1	CG2248	BKN28456
Rac2	CG8556	BKN30237
Rho1	CG8416	BKN28182
Sac1	CG9128	BKN20150
SCAR	CG4636	BKN22532
Shark	CG18247	BKN22750
Shibire	CG18102	BKN21495
Short stop	CG18076	BKN21543
Skittles	CG9985	BKN24385
SRA-1	CG4931	BKN21483
Syb	CG12210	BKN33351

Mbc	CG10379	BKN28072
Mcr	CG7586	BKN25702
Mdr65	CG10181	BKN26133
Mig-2-like	CG5588	BKN31897
MMP-1	CG4859	BKN28894
myospheroid	CG1560	BKN20614
NIMA	CG31765	BKN25308
NimB1	CG33119	BKN23336
NimB2	CG31839	BKN26789
NimB3	CG34003	BKN41810

TEP1	CG18096	BKN25880
TEP2	CG7052	BKN21154
TEP4	CG10363	BKN26228
TIMP	CG6281	BKN28268
Trio	CG18214	BKN28958
Vav	CG7893	BKN27749
Vha14	CG8210	BKN22163
W	CG2759	BKN29569
zCOP	CG3948	BKN28399
α-Adaptin	CG4260	BKN20148

For reamplification of dsRNAs from DNA templates in 96-well plates the standard Boutros Lab protocol was used: http://b110-wiki.dkfz.de/signaling/wiki/display/rnaiwiki/Protocols+RNAi+library+generation

Secondary RNAs were designed using the eRNAi tool from the Boutros Lab (http://www.dkfz.de/signaling/e-rnai3//). This algorithm avoids low-complexity regions in the target sequence for probe design and includes predictions regarding the specificity and efficiency of a probe allowing to estimate and avoid OTE. Probes were generated as described by Kennerdell & Carthew (1998). dsRNA probes were synthesized from PCR products of the respective cDNAs with T7 promoter sequences added on the 5' and 3' of the PCR primers.

Table 5: Secondary dsRNA primers.

T7 sequence was added 5' of each primer

Cdc42 fw	TGCCCGAGATTACACACCAT
Cdc42 rev	CGAGCACTCCACGTACTTGAC
Drpr fw	GCGGTGGTGTGCGTACGCAAATAT
Drpr rev	GGCAACATGGTGGCAGTGGATT
Dscam fw	AGGAGAACCCGCCGTACT
Dscam rev	GTGCGCTTGATCGACAGACT
Dscam fw	TGCTGGAGACACTGAAGGAA
Dscam rev	AGGCTAGGCAAAGGATGAGTT
Mdr65 fw	GGTGTGCGCTATCGAGGTAT
Mdr65 rev	TCATCGGGATCATAGTAACGC
Pi3K68D fw	CCCATTGGTCTTCTGGAGTG

Pi3K68D rev	CTCATCATCCCGCTCAGTTC
Rac2 fw	CGGGCCCAATTAATTCATAA
Rac2 rev	CACACACACACACATTCAAGC
MEGALIN fw	TATTGGCCACCAGTTAAGCG
MEGALIN rev	CACTTCCATTGCGCGTATAG
Syb fw	TTTTCCCATACTTCCGCCAC
Syb rev	ACCTTCTCCACGTTCACACG
Gliolectin fw	AGACCACAACAACAAAAGCAACCCCAG
Gliolectin rev	AGACCACTGTGTGTTTGTTTGTGGGCT
Annexin IX fw	AGACCACAACCAACAACAGGAGGATGC
Annexin IX rev	AGACCACCAGCTTTGCCGTTGAGTGTA
PGRP-LC fw	AGACCACCAACGAAGGAAGTCTGCTCC
PGRP-LC rev	AGACCACGATAGGGGTTAGGTGGGGAA
Mcr fw	AGACCACTATTACGCCAGGGTTTAATGGAAGT
Mcr rev	AGACCACGCTGGTTAATCTTCCACACTACAAT
TEPII fw	AGACCACCAGCCTGTTTTGGGTACCT
TEPII rev	AGACCACTTCTTCACCACAACCTGATAG
MMP1 fw	AGACCACCTTCTATCGCGGCTTTGAAC
MMP1 rev	AGACCACAACTTGCTGCCCTTGAAGAA
Puckered fw	AGACCACACCAACCACAAAGCGAAAAC
Puckered rev	AGACCACTAGCATTCGCGTTACACTGC
GFP fw	AGTGGAGAGGGTGAAGGTGA
GFP rev	AAAGGGCAGATTGTGTGGAC

RT-PCR/ *in situ* primers:

NIMA fw	CCGGAGGTATCACCACAAAAATGC
NIMA rev	GGCATAGGCGTAAGGTGGGGTTTT
NimB1 fw	TCTACTGACGCTGGTGGCATTTCC
NimB1 rev	GCAATCGGACAGGTGTGAATACAGG
NimB2 fw	ACCAGCAGAACCATGTGGAACAGG
NimB2 rev	tCATAGCCATCGCAGCAGACTTGG
NimB3 fw	GCACTTGACATCCACGCTGATTGG
NimB3 rev	AGCAAATGGGCTCGCACTTCAGG
NimB4 fw	GCTGCAACTTCACGAGCAACAGC
NmB4 rev	CCCACAATCTTCACCTCCTTGTCG
NimB5 fw	GGACCAGCCAACTTCCAAGATCC
NimB5 rev	TCGCACTCACCAGGAATCTTACAGC
NIMC1 fw	CCCATCGGCTTGAATGTTTGTAACC
NIMC1 rev	GCAAGTATTGGGGGACGAGCAGAA
NimC2 fw	AGTTCTCCTGGCCGCTGTGAGTGc
NimC2 rev	GCCCATCGTTTGATAAAGCCCAGA
NimC3 fw	tGTATCCCATGCTGGTCCTCGTCC
NimC3 rev	CCTCCATGCTCATCTCGATCTGG

PGRP-LA fw	GCACCATTCAGGATTCAGCCATTG
PGRP-LA rev	GCCAGCAGAAATTGGACACCTTCC
PGRP-LE fw	ACGCTCCCAAAACTCCGACACATT
PGRP-LE rev	TCTTACGTTGATCGCCCGCTTTTC
PGRP-LF fw	ATCACACGGCAACCGAAGGATG
PGRP-LF rev	GGGGCTTAATTGTCGATGGGCATA
PGRP-SB1 fw	TTGTGGCCGCTTTAGTGCTTTGCT
PGRP-SB1 rev	CGACCCTTGTGATCCGACTGAATG
PGRP-SB2 fw	CTTCAATCCGCATCAGTGCCAGTT
PGRP-SB2 rev	CACGGAATAGTTGGGCGAAACCTG
PGRP-SC1a fw	TGGTTTCCAAAGTGGCTCTCCTCCT
PGRP-SC1a rev	ATCATGTTCGGCTCCAGGGTGTC
PGRP-SD fw	AATCGCTGTCCAGGGGGAAGTACC
PGRP-SD rev	GGGCCACTGCTGTATCAGAGCGTA
PGRP-LC fw	CGCTCAAATATCAAACGAGCTGCAA
PGRP-LC rev	TATAACGCCCGAGTCTGTGGACGA
PGRP-SA fw	CGCTATGTGGTCATCCATCACACG
PGRP-SA rev	ATAAAGGCTATGCCCGTGCCAATG

qRT-PCR primers:

Cdc42 fw	CCTTCGAGAACGTCAAGGAG
Cdc42 rev	GTGATGGGCTTCTGCTTGTT
Malvolio fw	GGACACAAACAAGGCTACCG
Malvolio rev	ATGCCGCACAATCTCTACCT
Syb fw	CCACGTTCACACGCATAATC
Syb fw	AGAAGAAGCTGCAGCAGACC
zCOP fw	AATGGGAATGTCATCGTTGC
zCOP rev	GCTGGAAAACCTGGAGATCA
PSR fw	ATGACAAACCAGCGGTAAGG
PSR rev	TATTTTCGCGACGACCTCTT
DSCAM fw	GGTCTGGTTCACGGGTTCTA
DSCAM rev	CACCTACAACATTCGCATCG
rp49 fw	GATGCCCAACATCGGTTACG
rp49 rev	TTGTGCACCAGGAACTTCTT
GFP fw	AGTGGAGAGGGTGAAGGTGA
GFP rev	GTTGGCCATGGAACAGGTAG

8.6 Total RNA isolation and cDNA synthesis

Total RNA was isolated from $2x10^5$ S2 cells using the RNEasy kit (Qiagen). First strand cDNA synthesis was performed using 100 µg total RNA and the iScript kit (Biorad) according to the manufacturer's instructions. We isolated total RNA from S2

cells that had been treated with RNAi against specific genes, mock treated with RNAi against *gfp* or left untreated.

8.7 qRT-PCR

qRT-PCR experiments and analyses were performed as described by Arany, 2008 using Sso Fast Eva Green Supermix (Biorad) and the CFX96 thermal cycler (Biorad) and the primers listed in table 5. The amount of mRNA detected was normalized to control rp49 mRNA values. Normalized data were used to quantify the relative levels of a given mRNA according to cycling threshold analysis (ΔCt). Relative Δ Ct gene / Δ Ct rp49 ratios of untreated controls were anchored in 1 to indicate fold induction. Graphs represent the mean and SEM of relative ratios detected in 3 independent experiments.

8.8 Real-time PCR

Total RNA from all developmental stages of *Drosophila* was isolated using Trizol (Invitrogen) and the RNeasy kit (Qiagen). We used the iScript cDNA synthesis kit (Biorad) for reverse transcription and performed touchdown PCR with the primers in Table 5 and cycle numbers between 20 and 25 to obtain semi-quantitative gene expression profiles.

8.9 Immunohistochemistry

Immunohistochemistry was performed using the following antibodies: rabbit anti-activated Caspase-3 (CM1; Cell Signaling Technology; 1:25 or Santa Cruz, 1:50), mouse anti-GFP (Molecular Pobes, 1:50). Fluorescent secondary antibodies against mouse, rabbit, rat, guinea pig (Alexa Fluor 488, Cy3 and Alexa Fluor 568, Jackson ImmunoResearch; Alexa Fluor 488, Molecular Probes) were all used at 1:200. DAB staining was carried out using Vectastain Elite kit (Vector Labs) and 1.5 mM $NiCl_2$ for signal intensification. All solutions and buffers were generated according to protocols in Sullivan et al. (2000). Embryos were dechorionated for 3' in 50% bleach (Chlorox), then fixed in 3.7% formaldehyde for 20-23'; washes were performed in PBS + 0.1% Triton- X100; unspecific staining was blocked using 10% normal serum and antibodies were diluted in PBS+ 0.1% Triton+ 5% normal serum. Primary antibodies

were incubated overnight at 4°C and secondaries 1 h at RT. Stained embryos were stored and mounted in 80% glycerol.

8.10 Imaging of embryos

All confocal images were acquired using a Zeiss LSM 510 upright or 710 inverted confocal microscope. 0.5 µm for the stage 16 nervous system imaging and 1.14 µm confocal sections for the stage 13 whole embryo images were taken and stacks of 35 (stage 16) or 50 (stage 13) sections generated; image analysis was performed using LSM 510 or Zen software (Zeiss), ImageJ (NIH, USA, http://rsb.info.nih.gov/ij/) and Imaris 4.0/ 7.0 and ImarisBatch 1.3 (Bitplane).

8.11 *In* vivo phagocytosis assay

To quantitate the number of apoptotic particles and their engulfment by macrophages, confocal stacks (35 sections; total 17 µm) were acquired from the neural cortex of stage 16 ventral nerve cords, where most apoptosis occurs and where cell body glia reside. To quantitate the number of apoptotic particles outside the CNS, stage 13 embryos were imaged from lateral to medial, excluding the nerve cord (confocal stacks with 50 sections; total 57 µm). Three-dimensional reconstructions were built and the number and volume of activated Caspase-positive particles measured with an appropriate isosurfacing threshold using Imaris and Imaris Batch software. All data were collected with identical software parameter settings. Statistical significance of differences between experiments was assessed by one-way ANOVA with Dunett's post hoc test, with n = 8–20.

8.12 Fly infections

COL strain *S. aureus* (generous gift from Alexander Tomasz, Rockefeller University) from frozen culture were grown over night in tryptic soy broth (BD Biosciences) and diluted to have the same concentration at 6 pm. The stock solution was diluted 1:100 to reach a final concentration of ca. $2x10^6$ c.f.u. (colony forming units) ml^{-1}. For *E. coli*, the standard laboratory strain DH10B was used. Bacteria were grown for 16 h, spun down and resuspended in 100 µl LB media to ca. $4x10^7$ c.f.u ml^{-1}. For injection, adult male flies were used (4–5 days old). Flies were anaesthetized with CO_2 and infected via injection in the dorsal thorax with a glass needle loaded with the

bacterial suspension. Flies were returned to standard fly culture vials with food and incubated at 25 °C. Flies were infected in batches of 30. Following infection the number of surviving flies was recorded at intervals. Experiments were repeated at least three times (total of 90 flies). For each genotype and experiment, statistical significance was evaluated using Cox regression analysis of survival and calculation of the relative risk of death.

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Publications

- Axelrod, S., Kurant, E., Gaul U. Functional Dissection of Phagocytosis during Nervous System Development and in the Immune System of *Drosophila melanogaster*. *In preparation*
- Kurant, E., Axelrod, S., Leaman D., Gaul U. The Novel Phagocytic Receptor Six- microns- under acts upstream of Draper in Phagocytosis of Apoptotic Cells. *Cell, 2008*
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Selbstständigkeitserklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe, wirkliche und sinngemäße Zitate als solche gekennzeichnet und diese Arbeit noch nicht anderwertig zu Prüfungszwecken vorgelegt habe.

Berlin, den 22.11.2011

Sofia Axelrod